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ON FATTY ACID AND PHOSPHOLIPID 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

David Wilson Deamer, Jr., B.Sc., M.Sc.

* * * * * * *

The Ohio State University
1965

Approved by

David G. Cornell
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CONTENTS

ACKNOWLEDGMENTS ............................................................................ ii

FIGURES ........................................................................................................ v

TABLES ........................................................................................................... vii

ABBREVIATIONS AND SYMBOLS .............................................................. viii

INTRODUCTION AND HISTORICAL REVIEW ............................................. 1

The Concept of Cell Membranes ............................................. 1
The General Structure of Cell Membranes ................................. 2
Chemical Nature of the Membrane ............................................. 6
The Erythrocyte Membrane .............................................................. 7
The Myelin Sheath ............................................................................. 11
The Mitochondrial Membrane ......................................................... 12
Current Membrane Problems in Terms of Biological Functions ................. 14
The Lipophilic Layer ........................................................................ 14
The Hydrophilic Layer ................................................................. 17
Calcium Interactions with Membranes ........................................... 18
Calcium Interactions with Monolayers ............................................ 21
Statement of Problem ......................................................................... 26

EXPERIMENTAL MATERIALS AND METHODS ....................................... 28

Surface Pressure .................................................. 28
Surface Viscosity ........................................................................ 34
Fatty Acids, Diglycerides, Diethers ............................................. 38
Phosphatides .................................................................................... 40

DISCUSSION OF RESULTS ................................................................. 43

Fatty Acid - Calcium Interaction ............................................... 43
Phospholipid - Calcium Interaction ............................................. 79

SUMMARY AND CONCLUSIONS ............................................................. 93

BIBLIOGRAPHY .................................................................................... 95
## FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cyclic and Hydrated Phospholipid Structure</td>
<td>24</td>
</tr>
<tr>
<td>2. Capillary Tensionometer</td>
<td>29</td>
</tr>
<tr>
<td>3. Wilhelmy Apparatus</td>
<td>33</td>
</tr>
<tr>
<td>4. Slit Viscometer</td>
<td>35</td>
</tr>
<tr>
<td>5. Lattice Structure of Soap Film</td>
<td>44</td>
</tr>
<tr>
<td>6. Molecular Model of Calcium Stearate Disoap</td>
<td>46</td>
</tr>
<tr>
<td>7. Molecular Model of 1,3-Distearin</td>
<td>47</td>
</tr>
<tr>
<td>8. Molecular Model of 1,2-Glycerol Distearyl Ether</td>
<td>48</td>
</tr>
<tr>
<td>9. Film Viscosities</td>
<td>49</td>
</tr>
<tr>
<td>10. Stearic Acid on Water</td>
<td>51</td>
</tr>
<tr>
<td>11. Octadecanol on Water</td>
<td>53</td>
</tr>
<tr>
<td>12. Molecular Model of Type A Packing</td>
<td>55</td>
</tr>
<tr>
<td>13. Molecular Model of Type B Packing</td>
<td>56</td>
</tr>
<tr>
<td>14. Molecular Model of Type C Packing</td>
<td>57</td>
</tr>
<tr>
<td>15. Stearic Acid on Calcium Solutions</td>
<td>59</td>
</tr>
<tr>
<td>16. Stearic Acid on Calcium Solutions of Varying Concentration</td>
<td>60</td>
</tr>
<tr>
<td>17. Stearic Acid on Calcium Solutions of Varying pH</td>
<td>61</td>
</tr>
<tr>
<td>18. Stearic Acid on Solutions of Alkaline Earth Ion, pH 9.0</td>
<td>62</td>
</tr>
<tr>
<td>19. Stearic Acid on Solutions of Alkaline Earth Ions, pH 7.5</td>
<td>63</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>20.</td>
<td>Stearic Acid on Solutions of Alkaline Earth Ions, pH 6.0</td>
</tr>
<tr>
<td>21.</td>
<td>Stearic Acid, Distearin and Glycerol Dioctadecyl Ether on Water</td>
</tr>
<tr>
<td>22.</td>
<td>Oleic Acid, Elaidic Acid and Stearic Acid on Water</td>
</tr>
<tr>
<td>23.</td>
<td>Oleic Acid on Calcium Solutions</td>
</tr>
<tr>
<td>24.</td>
<td>Elaidic Acid on Calcium Solutions</td>
</tr>
<tr>
<td>25.</td>
<td>Viscosity of Dipalmitin and Distearin</td>
</tr>
<tr>
<td>26.</td>
<td>Surface Phase Transition Temperatures for Stearic Acid on Alkaline Earth Ions</td>
</tr>
<tr>
<td>27.</td>
<td>Molecular Model of Lattice Structure</td>
</tr>
<tr>
<td>28.</td>
<td>Phosphatidyl Choline on Water, pH 7.0</td>
</tr>
<tr>
<td>29.</td>
<td>Phosphatidyl Choline on Water, Varying pH</td>
</tr>
<tr>
<td>30.</td>
<td>Phosphatidyl Choline on Calcium Solutions</td>
</tr>
<tr>
<td>31.</td>
<td>Cholesterol on Water</td>
</tr>
<tr>
<td>32.</td>
<td>PC:Cholesterol on Water</td>
</tr>
<tr>
<td>33.</td>
<td>PC:Cholesterol on Water, Varying Temperature</td>
</tr>
<tr>
<td>34.</td>
<td>PC:Cholesterol on Calcium Solutions</td>
</tr>
<tr>
<td>35.</td>
<td>Phosphatidyl Serine on Calcium Solutions</td>
</tr>
<tr>
<td>36.</td>
<td>Calcium Interaction with Phosphate Groups</td>
</tr>
</tbody>
</table>
TABLES

Table

1. Lipids of Human Erythrocytes ................................................ 8
2. Phospholipids of Human Erythrocytes ................................. 8
3. Fatty Acid Distribution in Erythrocyte Phospholipids ...................... 8
4. Fatty Acid Distribution in Egg Phospholipid ................................ 10
5. Interspecies Variation in Erythrocyte Phospholipids ......................... 11
6. Lipids of Myelin ................................................................. 12
7. Lipids of Mitochondria ......................................................... 13
8. Properties of Bilayers .......................................................... 26
9. Correction Values of Viscometers ............................................. 37
10. Binding Strength of Alkaline Earth Ions ................................... 58
11. Surface Viscosities of Monolayers of Fatty Acids and Fatty Acid Derivatives 71
12. Bulk Phase Transition Temperatures ....................................... 73
13. Monolayer Phase Transition Temperatures ................................ 73
14. Binding Strength of Alkaline Earth Ions ................................... 74
15. Effect of Octadecanol Dilution .............................................. 77
16. Surface Viscosity of Phosphatides ......................................... 89
ABBREVIATIONS AND SYMBOLS

u - micron  
A - angstrom  
γ - surface tension  
π - surface pressure  
η - surface viscosity  
SA - stearic acid  
DP - dipalmitin  
DS - distearin  
PC - phosphatidyl choline  
PE - phosphatidyl ethanolamine  
PS - phosphatidyl serine  
PA - phosphatidic acid  
PI - phosphatidyl inositol  
PG - phosphatidyl glycerol  
SM - sphingomyelin  
TLC - thin layer chromatography
INTRODUCTION

The Concept of Cell Membranes

Membranes are probably the single ubiquitous structure of living organisms. In recent years it has become apparent that the cell and most of its components are defined by membranes, and that interaction between membrane constituents at the molecular level offers exciting possibilities for understanding many basic life phenomena.

Cell membranes have several important general capabilities:

1) They are the foundation for cell structure. An understanding of cell differentiation must ultimately derive in part from knowledge of how genetic material governs membranes.

2) They are selectively permeable when integrated with biochemical transport mechanisms.

3) They can build up and maintain an electrical potential, also in conjunction with biochemical reactions.

Homer Smith would place the discovery of the first cell membrane, the plasma membrane, with Carl Nageli (1). Nageli, who called it the Plasmamembran, first observed it in plant cells undergoing exosmosis. When algal cells were placed in a hypertonic solution, the cytoplasm could be seen to draw away from the cell wall and shrink. This could best be explained in terms of a semi-permeable membrane surrounding the cytoplasm from which water was being drawn by the difference in osmolarity.
Some years later, Pfeffer reached similar conclusions from a study comparing osmotic phenomena in plant cells with artificial osmometers (2). However, since the plasma membrane cannot be seen with the light microscope, the concept was doubted and debated during the next fifty years. In 1910, Höber began a long chain of studies which gave positive but indirect evidence for the existence of the membrane. He showed that whole erythrocytes have a very high resistance to electrical current even though their internal resistance is quite low (3). This result could only be explained by a layer of material around each cell which was relatively impermeable to the flow of ions.

Chambers, using micromanipulation techniques, was actually able to lift the membrane from the surface of an Arbacia (sea urchin) egg (4). He also demonstrated that many substances which cannot pass into the cell will diffuse freely within the cytoplasm if injected beneath the cell surface.

The gradual accumulation of indirect evidence was climaxed in recent years by direct observation of the postulated membrane together with the discovery of the membranous character of most other cellular structures.

The general structure of cell membranes

The lipid nature of the plasma membrane was suspected even while its existence was still in doubt. Overton, in 1899, had shown that the more "lipid soluble" a substance was, the more easily it entered a cell (5). Molecules such as lecithin and cholesterol could easily pass into cells, whereas much smaller molecules less soluble in lipid could not. Furthermore, lipid solvents were very powerful lysing agents
Gorter and Grendel (6) were among the first to experiment with physical measurements of known membrane systems, using the Langmuir monolayer technique which was relatively new at that time. They dissolved the lipid components of red blood cells from various sources and spread these on a Langmuir trough, measuring the surface area occupied by the monolayer when it first began to produce surface pressure. This area was then compared with the calculated total surface area of the number of erythrocytes which had been extracted. The ratio for dog, sheep, rabbit, guinea pig, goat and human cells was always approximately 2:1. Assuming that all the lipid was in the membrane, they concluded that the lipid was present as a bimolecular layer at the cell surface.

However, there are a number of objections to this experiment. The lipids were extracted with acetone, which does not readily dissolve phospholipids, one of the major membrane components. Dried erythrocytes were used to measure cell diameters, from which surface area was calculated by Knoll's formula: \( A = 2D^2 \). This is known to give much too low a value for surface area (7). For instance, Gorter and Grendel used 99 \( \mu^2 \) as the human RBC surface area, and more modern measurements give 160-170 \( \mu^2 \) (8). Furthermore, they have been criticized for using the area occupied by the film at zero surface pressure. Dervichian and Macheboeuf (9) repeated the experiment in 1938, extracting with ether-ethanol and compressing the monolayer to its collapse point before measuring the area. They also used better values for the surface area of erythrocytes. From their data, they concluded that only enough lipid was present to form a monomolecular layer at the cell surface.
It is obvious that the main problem with this experiment is the choice of surface pressure corresponding best to the molecular packing area in the red cell membrane. Egg phosphatidyl choline, for instance, first shows surface pressure at about 150 Å²/molecule and can be compressed to 65 Å²/molecule before the film collapses.

Some insight into the problem can be gained by using known values for phospholipid and cholesterol content of erythrocytes and comparing their molecular "areas" to the known area of the erythrocyte in the following manner:

1) Membrane lipids per cell (10).
\[ 1.3 \times 10^{-11} \text{ mg P/cell} \]
\[ 1.26 \times 10^{-10} \text{ mg cholesterol/cell} \]

Other lipids, such as free fatty acid and triglyceride, could only compose 2-3% of the cell surface.

2) Human red cell surface area is taken as 165 ± 5 μ² (8).

3) Cholesterol has a constant area in monolayers of 38-39 Å²/molecule and is essentially incompressible (11).

4) In the case of a monomolecular layer at the cell surface, such as that proposed by Dervichian, total area occupied by cholesterol is
\[ \frac{1.26 \times 10^{-13} \text{ g}}{387 \text{ g/mole}} \times 6.023 \times 10^{23} \text{ molecules/mole} \times 38 \text{ Å²/molecule} = 75 \mu^2. \]

Therefore 165-75 = 90 μ² occupied by phospholipid.

5) \[ \frac{1.3 \times 10^{-14} \text{ g}}{31 \text{ g/mole}} \times 6.023 \times 10^{23} \text{ molecules/mole} = 0.25 \times 10^9 \text{ molecules of phospholipid, assuming 1 P/PL.} \]
6) \( \frac{90 \times 10^8 A^2}{25 \times 10^9} = 36 A^2 / \text{molecule.} \)

7) In the case of a bilayer,
\( \frac{75 u^2}{2} = 37.5 u^2 \) occupied by cholesterol at the surface.

8) \( 165 - 37.5 = 126 u^2 \) occupied by phospholipid.

9) \( \frac{25 \times 10^7 \text{molecules}}{2} = 12.5 \times 10^7 \text{molecules PL.} \)

10) \( \frac{126 \times 10^8 A^2}{12.5 \text{molecules}} = 100 A^2 / \text{molecule.} \)

It is known from monolayer studies that even saturated phospholipids can occupy no less than 40 \( A^2 / \) molecule (12). Unsaturated phospholipids typically occupy 65-70 \( A^2 / \) molecule at the collapse pressure. Therefore something greater than a monolayer must be present at the red cell surface. The value of 100 \( A^2 / \) molecule for phospholipid in the case of the bilayer above is fairly typical of unsaturated phospholipids in mixed phospholipid: cholesterol monolayers at low surface pressure (13). It would seem that Gorter and Grendel’s conclusion was correct, with their incomplete extractions being balanced by too small an estimate of cell surface area.

Davson and Danielli, on the basis of this work and their own, published their concept of the cell membrane’s structure in 1935 (14). This has withstood many experimental tests and is now generally accepted as a working hypothesis. The proposed membrane was a "bimolecular lipid leaflet," the hydrophobic hydrocarbon tails of the lipids projecting inward, the hydrophilic groups interacting with a protein layer and water on the outer and inner surface of the membrane. This structure has come to be called the "unit membrane."
Probably the most important direct evidence in favor of this structure are the abundant electron micrographs of just such a double-layered membrane surrounding the cell and most cellular constituents. This has been demonstrated in the membranes of erythrocytes (15), amoeba (16), myelin sheaths (17), mitochondria (18), endoplasmic reticulum (19), nuclei (20), chloroplasts (21) and phospholipid preparations (22). However, all of these findings are based on dehydrated specimens and may not be a true representation of the membrane as it exists in the hydrated state.

Chemical nature of the membrane

The concept of biological membranes as "bimolecular lipid leaflets" is generally accepted. We are now faced with the more complex task of learning how the membrane components interact with each other and with their environment to produce the characteristic properties of the living cell membrane. Naturally it is necessary to determine the chemical nature of the membrane components, a study which is still going on.

It is a surprising fact that almost all that we know about membrane components is based on the study of only three sources: the erythrocyte, myelinated nerve tissue, and mitochondria. The problem is one of obtaining material which is known to come from a certain membrane and getting it in sufficient quantity. The three sources mentioned fulfill these requirements, as will be shown. Relatively little is known about the composition of the membranes of plant cells, mammalian tissue cells other than nervous and red cells, and the cell membranes of lower phyla, mainly because of the
difficulty of procuring a pure membrane preparation. Since membrane composition varies from tissue to tissue in a single animal, doubtless surprising variations in composition will be discovered as more sophisticated techniques allow us to examine a broader range of membranous material.

The Erythrocyte Membrane

The erythrocyte offers a particularly good source of membrane material because of several factors:

1) The cell is relatively simple, appearing to be little more than a bag of hemoglobin. There are no internal structures whose membranes would contaminate the plasma membrane components upon extraction (23).

2) The membrane can be prepared as a "ghost" in relatively pure form.

3) Red cells are one of the most easily obtained mammalian tissues and available in large quantities.

A large number of studies have been made on composition of erythrocyte membranes from various sources and these have been reviewed in an excellent article by van Deenen (24). The human erythrocyte membrane is fairly typical; the tables below give its composition as determined in a recent, rigorous study by Hanahan (10).
Table 1. Lipids of Human Erythrocytes

<table>
<thead>
<tr>
<th>Per cell values</th>
<th>Mean</th>
<th>Range</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipid (10^{-10}mg)</td>
<td>4.88</td>
<td>4.6-5.3</td>
<td>9</td>
</tr>
<tr>
<td>Lipid phosphorous (10^{-11}mg)</td>
<td>1.27</td>
<td>1.2-1.35</td>
<td>18</td>
</tr>
<tr>
<td>Cholesterol (10^{-10}mg)</td>
<td>1.26</td>
<td>1.12-1.42</td>
<td>16</td>
</tr>
<tr>
<td>Plasmologen (10^{-11}mole)</td>
<td>5.24</td>
<td>4.65-5.85</td>
<td>10</td>
</tr>
<tr>
<td>FFA (10^{-14}meq)</td>
<td>2.7</td>
<td>1.06-3.80</td>
<td>8</td>
</tr>
</tbody>
</table>

The lipid phosphorous is present in the form of phospholipid, with the following percentage distribution:

Table 2. Phospholipids of Human Erythrocytes

<table>
<thead>
<tr>
<th>SM</th>
<th>PC</th>
<th>PE</th>
<th>PS</th>
<th>PI</th>
<th>Lyso</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>23.8</td>
<td>29.5</td>
<td>25.7</td>
<td>15.0</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>Range</td>
<td>21.8-26.0</td>
<td>27.5-</td>
<td>21.5-</td>
<td>11.4-</td>
<td>1.2-</td>
<td>0-</td>
</tr>
<tr>
<td></td>
<td>30.9</td>
<td>28.9</td>
<td>17.6</td>
<td>4.2</td>
<td>1.6</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Each phospholipid has a characteristic distribution of fatty acids:

Table 3. Phospholipid Fatty Acid Distribution

<table>
<thead>
<tr>
<th>PE</th>
<th>PS+PI</th>
<th>PC</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16:0</td>
<td>15.5</td>
<td>4.4</td>
<td>34.7</td>
</tr>
<tr>
<td>16:1</td>
<td>.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18:0</td>
<td>14.1</td>
<td>39.7</td>
<td>13.8</td>
</tr>
<tr>
<td>18:1</td>
<td>17.2</td>
<td>9.8</td>
<td>21.1</td>
</tr>
<tr>
<td>18:2</td>
<td>5.6</td>
<td>2.6</td>
<td>21.9</td>
</tr>
</tbody>
</table>
Table 3 - continued

<table>
<thead>
<tr>
<th>PE</th>
<th>PS</th>
<th>PI</th>
<th>PC</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:3</td>
<td>.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:0</td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>20:3</td>
<td>1.6</td>
<td>2.6</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>20:4</td>
<td>21.8</td>
<td>23.5</td>
<td>6.7</td>
<td>.1</td>
</tr>
<tr>
<td>20:5</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:0</td>
<td></td>
<td></td>
<td></td>
<td>8.0</td>
</tr>
<tr>
<td>22:4</td>
<td>7.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5</td>
<td>1.1</td>
<td>.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6</td>
<td>8.9</td>
<td>7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:0</td>
<td></td>
<td>4.1</td>
<td></td>
<td>15.0</td>
</tr>
<tr>
<td>24:1</td>
<td></td>
<td>3.7</td>
<td></td>
<td>15.5</td>
</tr>
</tbody>
</table>

Sphingomyelin is the most saturated, containing over 70% saturated fatty acid. Phosphatidyl choline, serine and inositol are approximately 50% saturated. Phosphatidyl ethanolamine is least saturated, having only 30%. SM is characterized by its large content of 22:0, 24:0 and 24:1 acids, PC by its 18:2 content, PS and PI by their 18:0 content and PE by its generally large proportion of unsaturated fatty acids.

These values can be compared with egg phospholipids, with the fatty acid content compiled in the table below (25).
Table 4. Fatty Acid Distribution in Egg Phospholipid

<table>
<thead>
<tr>
<th></th>
<th>16</th>
<th>16:1</th>
<th>18</th>
<th>18:1</th>
<th>18:2</th>
<th>20:4</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>32</td>
<td>1</td>
<td>16</td>
<td>30</td>
<td>17</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>PE</td>
<td>19.8</td>
<td>1.2</td>
<td>39.5</td>
<td>20.1</td>
<td>9</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

(The last three figures in the PE values represent the total unsaturated fatty acids of 18, 20 and 22 carbon chain length.)

PC and PE compose 90% of the egg yolk phospholipid. Egg PC is quite similar to human erythrocyte PC in its fatty acid composition. Egg PE is much more saturated than erythrocyte PE. In contrast to erythrocyte and myelin phospholipid, egg phospholipid has almost no vinyl ether linkages. The significance of fatty acid distribution in membrane phospholipids is still unknown.

In the human red cell, assuming an average chain length of 18 carbons, the average molecular weight of the phospholipids is approximately 775. The total weight of phospholipid and cholesterol can then be calculated to be $4.76 \times 10^{-10} \text{mg}$. This is 97% of the total lipid present.

Although the human red cell is typical in terms of its qualitative make-up, the ratios of the various phospholipids vary greatly from one species to another. De Gier and Van Deenen (26) have shown one such series.
Table 5. Interspecies Variations in Erythrocyte Composition

<table>
<thead>
<tr>
<th></th>
<th>PC%</th>
<th>SM%</th>
<th>Cephalin%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>0</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>Ox</td>
<td>7</td>
<td>60</td>
<td>33</td>
</tr>
<tr>
<td>Pig</td>
<td>29</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>Man</td>
<td>36</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>Rabbit</td>
<td>44</td>
<td>33</td>
<td>24</td>
</tr>
<tr>
<td>Rat</td>
<td>58</td>
<td>23</td>
<td>19</td>
</tr>
</tbody>
</table>

The sphingomyelin and lecithin proportions vary inversely with each other, while the cephalin (PE and PS) fraction remains more or less constant. The PL/cholesterol ratio is approximately 1:1. Membrane permeability to glycerol in the above series increases with increasing PC content, one of the first instances in which a characteristic of a membrane has been linked with its composition.
The myelin sheath

The control and peripheral nervous system axons of vertebrates are surrounded by a substance called myelin. This material, when examined by electron microscopy, was found to consist of a large number of concentric layers of material showing the double-layer seen in other membranous cell constituents. It was later found that the myelin sheath was derived from the cell membrane by a "winding" process (27).

Since myelinated nerve tissue is abundant and the myelin readily separated by centrifugal techniques, it offers a second source of relatively pure membrane material for study. The composition of myelin has not been so well defined as the erythrocyte membrane. Nussbaum reported the following composition of rat brain myelin (28):

<table>
<thead>
<tr>
<th>Lipids of Myelin</th>
<th>PC</th>
<th>PE</th>
<th>PS</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids</td>
<td>29.3%</td>
<td>63.0%</td>
<td>7.6%</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>(16.7% of total lipids.)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cholesterol and phospholipid compose about 60% of the total lipid. The rest is accounted for by cerebroside and cerebroside sulfate. This general pattern is also seen in other myelin sources studied, the dog, cat, rabbit and man (29).
The mitochondrial membrane

The mitochondrion has a more complex structure than the erythrocyte and myelin, since its functions are more numerous. Besides the outer membrane and internal membranous cristae, there are evidently numerous "elementary particles" attached to the cristae. Lipid is present in all three structures. Eighty to ninety per cent of the mitochondrial lipid seems to be phospholipid, the rest being accounted for by cholesterol and cerebroside. Cornwell and Horrocks compiled the following table of compositions (30).

<table>
<thead>
<tr>
<th>Source</th>
<th>PC</th>
<th>PE</th>
<th>Cardiolipin</th>
<th>PI</th>
<th>PG</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>49</td>
<td>30</td>
<td>12</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Rat Kidney</td>
<td>41</td>
<td>30</td>
<td>9</td>
<td>14</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Sheep heart</td>
<td>46</td>
<td>35</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Beef heart</td>
<td>38</td>
<td>37</td>
<td>16</td>
<td>6</td>
<td>1trace</td>
<td></td>
</tr>
<tr>
<td>Beef heart</td>
<td>41</td>
<td>33</td>
<td>15</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cardiolipin has so far been found only in mitochondria. It is interesting that there seems to be relatively little difference between species so far as mitochondrial PL ratios are concerned. Also, the very small quantity of PS should be noted since Tobias has suggested that PS may be the material which is active in calcium interactions with membranes (31). This certainly could not be true for mitochondria.
Summary

Cellular membranes are seen to be composed mainly of phospholipid, with varying amounts of cholesterol. The phospholipid composition varies from tissue to tissue and from species to species. Within each type of phospholipid from a single source, the fatty acid composition seems to be more or less characteristic, certainly not randomly distributed. The phospholipid and cholesterol in the ideal membrane are arranged in a double layer, with their hydrocarbon positions projecting inward and their hydrophilic groups (hydroxyls, phosphates, nitrogen bases and carboxyl groups) interacting with water, protein, and possibly each other.

Current membrane problems

A number of biological activities will probably be understood in terms of membrane structure and interactions at cell surfaces. These include the following:

1) The action of certain hormones, notably the steroids
2) Function of fat soluble vitamins
3) Selective permeability, active transport
4) Nerve impulse transmission
5) Integrity and form of cell structures

From the picture of general membrane structure which has been developed, there seem to be two portions of the membrane in which such interactions might occur: the hydrophobic layer and the hydrophilic layer.

The lipophilic layer

This consists of the hydrocarbon moieties of the amphipathic
membrane molecules and forms the major portion of the membrane. Interaction occurs between the chains in the form of London-van der Waal's dispersion forces, although the main force preserving the smectic form may be "hydrophobic" or entropic bonding. The hydrocarbon layer is responsible for the gross properties of the membrane, such as its impermeability to molecules having a diameter greater than 8-10 Å, its permeability to "lipid soluble" molecules, and its high electrical resistance.

The amount of interaction due to forces acting within the lipophilic layer can be varied in several ways:

1) Varying the length of the hydrocarbon chain. Shorter chains have less van der Waal's interaction. This is clearly demonstrated by the melting points of a homologous series of saturated fatty acids. As chain length increases, the melting point is raised. Vandenheuval has calculated the increase in bond energy to be \( \sim 1 \text{ Kcal/CH}_2 \) (32). Thus two straight 18 carbon chains have an attractive energy of \(-18 \text{ Kcal}\).

2) Addition of double bonds. Stearic acid melts at \(69^\circ\). Oleic acid, with a single \textit{cis} double bond at the 9 carbon, melts at \(14^\circ\). The kink in the chain caused by the presence of the double bond disallows close packing along the entire length of the chain. This effectively cuts the chain in half. (For instance, a 9 carbon straight chain fatty acid melts at \(18^\circ\).)

3) Addition or deletion of different types of molecules.

All of these possibilities must be taken into account in
attempting to understand the cell membrane. Van Deenen has made an important study of these variables, testing the effect of chain length, double bonds and addition of cholesterol in artificial "membranes" of synthetic phospholipids (33). In this work, monolayers of the materials were spread on a Langmuir trough and their surface pressure/molecular area curves were compared.

It was found that the type of hydrophilic head (choline, ethanolamine, serine or phosphate) had little effect on the curves of saturated phospholipid. These compounds all had a closest stable packing of 40-50 $A^2$/molecule. This would be predicted from a knowledge of monolayers of saturated fatty acids in which the closest packing is approximately 20 $A^2$/chain (34). Shortening the chain length of either or both hydrocarbon tails produced a less condensed film. For instance, dicapryl PC at a pressure of 20 dynes/cm had an area of 80 $A^2$/molecule, whereas dilauryl PC at the same pressure occupied 70 $A^2$. Distearyl PC had an area of 45 $A^2$.

Oleic acid monolayers are of the liquid-expanded type (34). Addition of oleic acid to the phospholipid at either position also expanded the monolayer. Oleyl stearyl and stearyl oleyl phosphatidyl choline both had an area of 60 $A^2$/molecule at 20 dynes.

The importance of these findings is obvious, since membrane phospholipids contain a large proportion of unsaturated fatty acids. The amount and nature of unsaturation certainly plays an important role in membrane properties. One indication of this is the finding that distearyl lecithin is a powerful lysing agent for red blood cells (35). The ratio of saturation/unsaturation is evidently very
critical. The fact that various unsaturated fatty acids are essential to different organisms also suggests that the unsaturation must be of a certain nature, assuming that these fatty acids are being used in cell membranes.

Van Deenen also confirmed with synthetic PC the finding that cholesterol has a condensing effect when present in a mixed monolayer. The area/molecule at 5 dynes/cm of a 1:1 cholesterol:PC (unsaturated) mixture was 65 A$^2$, when 80 A$^2$ would be expected by simple addition of areas. This has been noted several times previously (36, 37) and has been attributed by Dervichian to the formation of a 3:1 cholesterol:phospholipid complex (37). The effect is most apparent when the phospholipid is unsaturated.

The hydrophobic layer is the obvious place to look for interactions of certain vitamins and hormones. Wilmer (38) has noted the resemblance of estrogens, glucocorticoids, androgens and vitamin D to cholesterol and has proposed several possible modes by which they might replace cholesterol in the membrane and alter its functional structure.

The hydrophilic layer

This is actually two "layers", on the outer and inner surfaces of the membrane, and is made up principally of the charged groups of phospholipids and the hydroxyl groups of cholesterol. It is this layer which is probably functioning in the transfer of ions across the membrane, which in turn builds up the electrical potential of the cell.

As Vandenheuval has pointed out, the situation at the hydrophilic surface is exceedingly complex and oversimplification in various
studies has produced a large number of conflicting theories and speculations (32). To understand any single membrane phenomenon, the entire membrane must be considered. However, a number of these considerations are still outside the realm of our physical knowledge. A search for these fundamental facts must be the starting point in any attempt to understand the total membrane. One such area is the manner in which polyvalent cations, in particular calcium, alters the properties of membranes.

Calcium interactions with membranes

The importance of the calcium ion in biological processes is well known. Its presence is necessary in numerous enzyme systems, in blood clotting, for muscle contraction and for nerve impulse transmission. Its gross function appears to be as a biological "glue" in which its double positive charge interacts with two monovalent negatively charged oxygens, binding them together. It is aided in this by the partial covalent nature of the bond which is formed.

Its affect on membrane permeability has been fairly well studied in vivo, but its precise action is obscure. A clear understanding of this single effect may lead to a more complete concept of membrane structure as a whole.

As might be expected from the "glue" analogy, calcium is functional in preserving the integrity of many membranes. For instance, in a number of fish erythrocytes removal of calcium from the medium by addition of oxalate or fluoride causes hemolysis to occur (39, 40). Lyman (41) found the same effect for erythrocytes
of the snapping turtle in a calcium free medium. Magnesium, strontium and barium were not able to substitute for the calcium ion. Maisels (42) demonstrated that the red cells of the African tortoise had a similar calcium dependence, and that in the absence of calcium passive permeability to sodium increased 20 fold.

Heilbrun demonstrated that calcium is necessary for reforming the Arbacia egg membrane after injury (43). If calcium ion is not present in the environment, the cytoplasm simply flows out through the smallest cut. However, this effect may be due to a clotting action in which calcium is essential.

Although calcium is not necessary for the integrity of all erythrocyte membranes, permeability changes do occur if it is removed. For instance, Bolingbroke and Maizels demonstrated this in the human red cell in the following manner (44):

1) Red cells were incubated three hours in a 6 per cent lactose solution with no electrolytes present. The cells became highly permeable and electrolytes rapidly leaked out of the cells.

2) The cells were then placed in a KCl solution. Potassium ions entered the cells by passive diffusion. (The normal physiological state of human red cell interior concentrations is high potassium, low sodium.)

3) The cells were finally placed in 70 mM NaCl. Sodium ion exchanged with potassium at a rapid rate, a flux which does not occur in the normal cell. If, however, calcium was present, the impermeability of the cells was restored to normal and little influx of sodium occurred. Mg, Sr and Ba were quite ineffective in restoring the normal state.
Whittembury (45) has shown similar effects of calcium in a more quantitative manner. Using "probing molecules" of different molecular radii, he has determined the equivalent pore radius (EPR) of various tissue membranes. In Necturus kidney tissue, it was found that calcium concentrations between 1-10 mM has no effect on EPR, but that it is increased in the absence of calcium. For instance, with no calcium present sucrose enters the cell easily while normally it cannot. Its molecular radius is 4.5 Å. Raffinose, however, with a radius of 6.0 Å, is still excluded from the cell, indicating that calcium has a very precise control over the EPR.

A second role of calcium, certainly related to its function in permeability, is the flux that occurs during muscle fiber contraction and nerve impulse transmission.

Calcium has long been known to be necessary for muscle contraction. Ringer, in 1882 showed that the frog heart in isotonic NaCl stops beating (46). Addition of calcium ion will revive the heart, although the beat is still abnormal. Potassium is necessary to produce a normal beat.

It has been more recently demonstrated by several workers that the presence of potassium allows normal bioelectrical changes in muscle fibers but that no contractions are possible unless calcium is also present (47,48).

Whether the action of calcium in muscle contraction is a membrane phenomenon, or acting directly on the myofibrils, or both, is not yet clear. The situation is somewhat simpler in nerve impulse transmission, involving a flux of sodium and potassium across a membrane with a concurrent release of calcium. Hodgkin (49) demonstrated this effect in squid giant axon, where calcium influx in the resting state was found
to be .076 umoles/cm². This increased to .082 upon stimulation.
Koketsu found the reverse to be true with myelinated fibers, where an
efflux occurs with stimulation (50).

This flux has generally been interpreted as a loss of calcium
from the anionic groups of the nerve cell membrane, resulting in an
increased water content of the membrane phase. The increased hydration
and calcium displacement contribute to a "loosening" of the membrane
structural components, causing an increase in ionic permeability and
ultimately, depolarization.

**Calcium interactions with monolayers**

The precise manner in which calcium interacts with cell membranes
is still unknown. The most common interpretation is comparing it with
the effect of calcium on fatty acid monolayers. In 1917 Langmuir pub­
lished his first paper on monolayers in which he demonstrated that
numerous substances can form stable films a single molecule thick when
spread on water (51). One of his early findings was that various poly­
valent ions such as calcium, barium and aluminum caused the film to
become rigid, also altering the properties of their surface pressure/area
curves. This effect has been generally interpreted as the formation of
a calcium distearate soap which has a high intrinsic surface viscosity.

As was mentioned before, calcium may be affecting the membrane
in much the same manner. A number of workers have used the calcium
interaction with palmitic and stearic acid monolayers as a model of
what may be occurring at the cell surface.

Webb and Danielli compared palmitic acid monolayers to membranes,
testing the relative strength of bonding of sodium and calcium to anionic sites (52). They found that sodium could compete effectively only in concentrations 100 times that of the calcium ion. The Gibbs-Donnan relation for simple electrostatic attraction was not accurate in predicting this ratio, indicating that there is some covalency in the calcium-oxygen bond which forms.

Gershfeld and Shanes have used stearic acid monolayers to study binding of veratrine alkaloids in competition with calcium (53). Veratrine competes very effectively for sites, producing a gaseous film even in the presence of calcium. \textit{In vivo} veratrine has the effect of depolarizing nerve fibers and Shanes suggested an analogy between its affect on films and neuronal membranes.

Shanes also draws a parallel between membrane permeability and monolayer permeability (54). In this hypothesis, a mathematical model is shown to fit data from membranes and monolayers if "holes" are present, or conversely, if the membrane materials are present as "islands."

Abood et al. investigated N-methyl-2-pyrolidyl methyl cyclopentylphenyl glycolate (PMCG) with stearic acid monolayers (55). Since this drug resembled calcium in its effect on muscle, it was reasoned that a monolayer might also show similarities in their action. In fact, $10^{-5}$ M PMCG increases the rigidity of a stearic acid monolayer and also competes for sites on a lecithin film.

Rogeness and Abood compared the effects of the psychotomimetic drug N-methyl-3-piperidyl benzilate (NPB) and calcium on the surface properties of stearic acid (56). Again, surface pressure/
molecular area curves and surface viscosity alterations were similar. From these experiments it was concluded that certain drugs may act by competing with calcium for sites on the cell membrane.

Certainly the cell membrane is not composed of free fatty acids. To make a more exact model of the membrane a number of workers have used various phospholipid preparations and studied the effect of calcium on these. Alexander made one of the first studies of calcium interaction with phospholipid monolayers. Kephalin was spread at a benzene-water interface in the presence and absence of calcium in the aqueous phase. The calcium had the apparent effect of expanding the film. This was explained as an anchoring effect of calcium on the film, the salt remaining at the interface rather than dissolving in the benzene. From this result it was postulated that "in the cell membrane calcium ions 'pull' kephalin molecules into the interface, resulting in an increased molecular packing and lowered interfacial tension. The thus tightened membrane would be expected to show a generally decreased permeability." (57)

Kimizuka and Koketsu, using Ca$^{45}$, recently proved that .1 mM calcium binds to pure lecithin monolayers in a 1:2 ratio at physiological pH (58). EDTA, 5 mM, and 0.4 mM ATP were found to completely inhibit this absorption. K and Na (.1M) could partially displace the calcium ion.

Rosano and Shulman spread phosphatidyl ethanolamine as a "duplex film" and investigated calcium interactions. In this method, cephalin was mixed with N-hexadecane in a 1:35 mole ratio. When spread on .002 M NaCl saturated with N-pentanol the mixture formed a duplex film in which the PE film was penetrated by molecules of hexadecane and pentanol.
The pentanol was essential to the formation of the film. The value of such a method is that it duplicates the oil-water interface in an easily manageable form, which is probably more closely related to cell surfaces than a film at the air-water interface.

The area/cephalin molecule of the duplex film was 70 Å² at 40 dynes/cm. When 2 mM calcium was added to the system the film contracted to 50 Å²/molecule, expelling the penetrating molecules. When the solution was made up to 20 mM calcium, the film expanded to 100 Å²/molecule.

Rosano proposed a very interesting explanation for this latter effect. The most probable configuration of the hydrophilic tail of PE in a monolayer is the cyclic structure shown below:

![Figure 1. Cyclic and Hydrated Phospholipid Structure](image-url)
Rosano suggested that "calcium injected under cephalin monolayers breaks the phosphoric acid-amine polar group intramolecular association, permitting intermolecular association between neighboring cephalin molecules. This permits surface lattices to form. This again enables the oil molecules to penetrate and give pressure rises at constant area." (59)

Rojas and Tobias (60) have worked with monolayers of PC, PE, PI and PS at the air/water interface, comparing pressure/area curves with and without calcium being present in the subphase. They found that only PS showed any effects of calcium, its curve becoming somewhat more condensed.

Nash and Tobias (61) have developed a membrane model quite removed from monolayers. In this, a millipore filter is soaked in a benzene solution of phospholipid and cholesterol, then placed as a barrier between two cells. The effect of various ions on its electrical resistance can then be measured. Again, it was found that only PS showed an increase in resistance upon addition of calcium to the subphase.

Mueller has actually produced a bimolecular layer of extracted brain lipids by allowing a "bubble" of the extract to form across a small orifice (62). The variation in electrical resistance of these membranes with voltage in the presence of certain substances (as yet unknown) resembled results from similar experiments with living cell membranes.

Thompson has probably worked out the most elegant membrane model so far. (63) In this technique, a mixture of phospholipid and tetradecane in chloroform is painted across a 1 mm hole in a thin Teflon sheet. This is then placed as a barrier between two water
filled cells. As the solvent dissolves into the aqueous phase, the film thins and eventually forms a bimolecular membrane similar to Mueller's but with known components.

Properties of this membrane are similar to those of the living cell membrane.

Table 8. Properties of Bilayers

<table>
<thead>
<tr>
<th></th>
<th>Synthetic</th>
<th>Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness</td>
<td>61 ± 10A</td>
<td>75 A</td>
</tr>
<tr>
<td>Electrical resistance</td>
<td>(2.2-4) x 10^6 ohm cm^-2</td>
<td>10^3-10^5 ohms cm^-2</td>
</tr>
<tr>
<td>Surface tension</td>
<td>1.0 dyne cm^-1</td>
<td>.03-1 dyne cm^-1</td>
</tr>
<tr>
<td>Water permeability</td>
<td>4.4 ± .5 u min^-1 atm^-1</td>
<td>.1-3.0 u min^-1 atm^-1</td>
</tr>
</tbody>
</table>

One interesting finding here is the very low surface tension. The low tension of the cell membrane has long been interpreted as evidence for a protein film present at the cell surface. In view of Thompson's results, this no longer follows.

Statement of the problem

Fatty acid monolayers offer a simplified version of what may possibly be occurring when calcium binds to the cell surface. However, the nature of calcium interaction in fatty acid monolayers is still open to question. Since a number of workers are using this system as a cell membrane model, it is important to understand how closely it does in fact resemble the cell membrane.

Therefore, it was proposed to investigate this interaction. There are three possible explanations for the effect of calcium on fatty acid monolayers: 1) charge destruction; 2) formation of disoap molecules;
3) formation of a copolymeric soap lattice structure. (These are discussed in detail on page 43.)

The three effects can be differentiated using surface pressure/area, surface viscosity and surface phase transition temperature as parameters of molecular interaction. If charge destruction is important, destroying the charge on the film by other means should alter surface properties in a manner similar to calcium. If formation of a disoap molecule is the major contribution in the calcium effect, molecules with structures similar to that of the proposed disoap molecule should have surface properties comparable to fatty acid films on calcium solutions. If a soap lattice structure is being formed, its phase transition temperature should be much higher than that of the disoap analogs.

Since phospholipid is the main component of the cell membrane, and since calcium is known to interact with phospholipid monolayers, it should also be possible to apply the above techniques to phospholipid films. In particular it is important to further test Rosano's hypothesis about how calcium may be affecting phospholipid films, since such a rearrangement might help explain the presence of the nitrogen bases in phospholipid molecules. If the Rosano interaction is occurring, it should show up as a change in surface viscosity. Furthermore, this change should not occur when calcium interacts with phosphatidic acid, where the positive tail is absent.

Lastly, Tobias has suggested that only phosphatidyl serine may be important in calcium interactions at the cell surface. It is important to see if his results can be confirmed in surface viscosity studies of phosphatidyl serine monolayers.
Surface pressure

This is the classical tool in the study of monomolecular films. The term is actually something of a misnomer, since surface pressure arises from quite different causes than those usually associated with physical pressure. However, like three-dimensional pressure, it is a function of concentration and in this way is analogous to gaseous pressure.

Surface pressure ($\Pi$) arises from a difference in surface tension on opposite sides of a barrier and is defined as

$$\Pi = \gamma_{\text{H}_2\text{O}} - \gamma_m$$

where $\gamma_{\text{H}_2\text{O}}$ = the surface tension of water and $\gamma_m$ = the measured surface tension of the film covered surface.

Surface tension at any liquid interface arises from the attraction of the molecules in the bulk liquid for the molecules at the surface. This produces a resultant force in the plane of the surface which is proportional to the forces acting to bind the molecules together.

In hydrocarbons, the only forces acting are the weak London-van der Waal's dispersion forces which occur when one fluctuating dipole induces a dipole in a neighboring molecule. The resulting surface tension is relatively weak.

In materials like mercury and liquid metals, at the other end of the spectrum, strong metallic bonding is present in addition to dispersion forces and the surface tension is correspondingly very high.
In water, both hydrogen bonding and dispersion forces are acting. The surface tension of water lies between that of hydrocarbons and liquid metals. $\gamma_{\text{H}_2\text{O}} = 72$ dynes/cm, $\gamma_{\text{Hg}} = 484$ dynes/cm, and $\gamma_{\text{benzene}} = 28$ dynes/cm.

Surface tension is a true force and can do work when unbalanced. Therefore it can also be expressed as surface energy:

$$\text{dynes/cm} = \text{ergs/cm}^2 \quad (1 \text{ dyne} = 1 \text{ erg/cm})$$

One of the most accurate measures of $\gamma$ is made by allowing it to do work by lifting a column of water in a capillary tube:

![Fig. 2. Capillary Tensionometer](image)

Fig. 2. Capillary Tensionometer
1) Force holding water up = \[ 2\pi r \gamma \cos \theta \]

2) Force acting downward = \[ \pi r^2 \rho g \]
   \[ \rho = \text{density of water} \]
   \[ g = \text{gravitational acceleration} \]

3) \[ F_1 = F_2 \]
   \[ 2\pi r \gamma \cos \theta = \pi r^2 \rho g \]

4) \[ \theta \text{ usually is close to 0} \]
   \[ \therefore \theta = \frac{1}{2} \rho \]

A second, less accurate but more convenient way to measure is the Wilhelmy plate method (64). Here, a clean hydrophilic plate suspended from an analytical balance is dipped into the liquid. The same forces which draw water up a capillary column act to draw water up the side of the plate. The amount of water is "weighed" by adjusting the balance and this is proportional to the surface tension, as proved by Allan (65).

Amphipathic compounds are materials with a hydrophilic and hydrophobic groups present on the same molecule. Examples are the various soap and detergent molecules, phospholipids, cholesterol and proteins. These compounds, under the proper conditions, will spread on water to form a monomolecular film with the hydrophilic heads "dissolved" in the water and the tails on the surface. When the molecules are close enough together, the tails align themselves more or less perpendicular to the surface, forming a two dimensional crystalline structure. Since the van der Waal's forces acting in the tails are weaker than the hydrogen bonds of the water's surface, once the surface is covered with the film its surface tension is lowered, eventually approaching that of a liquid hydrocarbon as compression is increased.
If a surface of water is cleaned and a barrier placed across the surface, a film can be spread such that the barrier separates clean surface from film-covered surface. Since the clean surface retains its $\gamma$ of 72 dynes/cm while the film-covered surface has $\gamma = 42$ dynes/cm, an unbalanced force results. This force is surface pressure:

$$\pi = 72 - 42 = 30 \text{ dynes/cm}$$

Langmuir devised an ingenious technique for measuring $\pi$ (51). This consisted of a long, paraffined trough containing water on which a film could be spread. A floating mica barrier was placed across one end, producing a patch of clean water surface. The mica was linked to a torsion balance, enabling $\pi$ to be measured in dynes/cm. A known amount of surface active material, such as stearic or oleic acid, was spread on the water beyond the mica barrier and slowly compressed. At a certain area per molecule, surface pressure began to register on the balance. As area was further decreased, the force built up to approximately 40 dynes/cm and the film collapsed. Surface pressure could be plotted against area per molecule to give curves analogous to pressure/volume isotherms for gases.

The Wilhelmy plate can also be used for measuring $\pi$ on a Langmuir trough arrangement and is probably the method of choice, since it is easily more sensitive than the barrier-torsion balance linkage by a power of ten. A modification of this method was used in the present study.

In this instrument, the plate is set up as though to measure $\pi$ except that it dips into a Langmuir trough. The balance is zeroed
with a clean water surface and the film is spread and compressed. As $\gamma$ decreases, the amount of water held up by the plate diminishes proportionately and the plate rises out of the water. Classically, this rise is measured by an optical lever attached to the balance beam. However, besides being cumbersome, a correction must be made for the change in buoyancy. In the present modification a null point technique was used. The beam was allowed only a slight motion during compression, then brought back to a null point by adjusting the weights on the balance. The balance motion was magnified by a microscope focussed on the tip of the balance needle, rather than by an optical lever. A fine glass hair was attached to the end of the needle to allow greater accuracy in measuring. The apparatus is diagrammed in Figure 3.

A sample calculation of $\Pi$ is shown below:

\begin{align*}
\text{Mg at start (clean surface)} & = 500 \text{ mg} \\
\text{Mg with film present} & = 400 \text{ mg} \quad \Delta = 100 \text{ mg} \\
1 \text{ mg} & = 0.98 \text{ dynes} \quad \Delta = 98 \text{ dynes} \\
\text{Plate perimeter} & = 4 \text{ cm} \\
\Pi & = 98/4 = 24.5 \text{ dynes/cm}
\end{align*}

The trough itself was milled from a piece of solid Teflon, since earlier experience showed that paraffin readily contaminated the water. Use of Teflon also completely negated the possibility of metallic ions entering the subphase. The water space measured 10x50x1 cm, and contained approximately 500 ml. The dipping plate was a 1" square of platinum foil which was cleaned after each run by dipping in chromic acid cleaning solution. Before each day's run it was made completely hydrophilic by being heated to red heat in a Bunsen burner flame. The barriers were
thermis
temp
to heating
unit

microscope

plate
threaded
rod

motor

rheostat

Figure 3. Wilhelmy Apparatus
made of Lucite plastic strips, measuring 3 x 14 x .5 cm. They were
driven during compression by a threaded rod passing through a brass
bar fastened to the barrier. The rod was turned either by hand or by
a 5 RPM electric motor. Various pulley ratios allowed the barriers to
be driven at any desired speed.

The trough and plate were enclosed in a vinyl plastic case to
protect against drafts and dust. The case was thermostated by a
YSI Thermistemp (Yellow Springs, Ohio) which could vary the temperature
from 15° to 45° when so desired.

Surface viscosity

Surface viscosity is a second useful parameter of molecular inter-
action. It is particularly valuable when measured as a function of
temperature, since this gives some idea of the type and extent of forces
acting between molecules, just as melting points of matter in the bulk
phase indicates the strength of interaction.

There are a number of techniques by which surface viscosity may be
measured. These include rotating disk (66), oscillating disk (67),
viscous traction (68), canal and slit viscometers (69) and the simple
talc test. The most commonly used are oscillating disk and canal or
slit viscometers. The former are more adaptable to work with high
viscosity films, the latter to low viscosity films. The slit viscometer
was chosen for the present work.

The viscometer was made from a modified plastic barrier. A bridge
opening was cut into the center of the barrier. Two glass cover slips
(2 x 5 cm) were fastened so that a narrow adjustable channel of open
water was formed beneath the bridged barrier. Film flow could occur
through the channel.

In use, a monolayer was spread as usual and compressed by the drive mechanism to the desired surface pressure. Since film was flowing through the slit, continuous motion of the barrier was necessary to maintain the pressure. The rate of change of area on the film side was measured and this was equivalent to the area of film which flowed through the slit.

None of the surface viscometers are completely satisfactory. Only the theory of the canal and slit viscometer can be derived a priori and a number of corrections and assumptions must still be made. A simple derivation for slit viscometry is a two-dimensional version of the Poiseuille theory for the capillary viscometer:

\[ \text{Fig. 4. Slit Viscometer} \]
1) Definition of $\eta_s$: If two surfaces move past each other at 1 cm/sec and are 1 cm apart, and if 1 dyne of tangential force is found on 1 cm of length of one surface, $\eta_s = 1$ surface poise. 

\[
\text{Force} = \frac{\eta_s \times \text{length}}{\text{width} \times \text{velocity}}
\]

\[\therefore \eta_s = \text{dyne sec/cm} \]

2) Derivation for slit viscometer:

Force acting on aa (in above diagram) is equal to $2rP$, where $P$ is the surface pressure/cm.

3) Force resisting motion of rectangle $aaa'a'$, from definition of $\eta_s$ is equal to $2L\eta_s \frac{dv}{dr}$

4) These forces (2 and 3) are equal and opposite:

\[F_p = F_s\]

\[\therefore 2rP = 2L\eta_s \frac{dv}{dr}\]

5) \[\frac{dv}{dr} = -\frac{rP}{L\eta_s}\]

6) Integrate 5:

\[v = -\frac{r^2P}{2L\eta_s} + C\]

7) At $V = 0$, $r = C$

8) \[0 = -\frac{r^2P}{2L\eta_s} + C\] or \[C = \frac{r^2P}{2L\eta_s}\]

9) \[V = \frac{P}{2L\eta_s} (R^2 - r^2)\]

This is a parabola of form $V = K - Kr^2$ where $K$ = a constant.

10) Area of parabola = area of film which passes through slit in unit time.
\[ \frac{dA}{dt} = \frac{\text{Area}}{\text{Time}} = 2 \int_0^R \frac{P}{2L \eta_s} (R^2 - r^2)\,dr \]

\[ 2 \left[ \int_0^R \frac{PR^2}{2L \eta_s} \,dr - \int_0^R \frac{PR^2}{2L \eta_s} \,dr \right] = \]

\[ 2 \left[ \frac{PR^3}{2L \eta_s} - \frac{PR^3}{3.2L \eta_s} \right] = \frac{2}{3} \frac{PR^3}{L \eta_s} \]

11) \( R^3 = \left( \frac{1}{2} D \right)^3 = \frac{1}{8} D^3 \)

\[ \frac{dA}{dT} = \frac{2}{3} \cdot \frac{1}{8} \frac{PD^3}{L \eta_s} = \frac{\pi D^3}{12L \eta_s} \]

12) \( \eta_s = \frac{\pi D^3}{12L} \frac{dA}{dT} \)

There are two important theoretical drawbacks to this simplified theory:

1) The underlying water exerts a drag on the moving film and thus affects the measured viscosity. This is a problem with every type of surface viscometer. Harkins (70) and Joly (71) have derived correction factors for this drag:

Table 9. Correction Values of Viscometer

<table>
<thead>
<tr>
<th>Slit</th>
<th>Factor</th>
<th>Slit</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mm</td>
<td>0.00032</td>
<td>1mm</td>
<td>0.0003</td>
</tr>
<tr>
<td>0.5mm</td>
<td>0.00016</td>
<td>0.5mm</td>
<td>0.0001</td>
</tr>
<tr>
<td>0.1mm</td>
<td>0.00003</td>
<td>0.1mm</td>
<td>0.00001</td>
</tr>
</tbody>
</table>

These factors are not very satisfactory, however. At high viscosities they are insignificant and at low viscosities their application
often results in negative values. We have therefore used the uncorrected version of the surface viscosity equation.

2) There is necessarily a difference in $\Pi$ across the slit. This means that as the film passes through the slit, the pressure acting upon it falls to zero. Since $\eta_s$ is a function of $\Pi$, a large pressure differential will produce low values for $\eta_s$.

However, in the present study we were interested in relative surface viscosity changes, rather than absolute values. For this purpose the slit viscometer was entirely adequate.

Surface phase transitions with temperature variations could theoretically be determined in the slit viscometer, measuring viscosity as the temperature is increased. However, in the present apparatus, the highest temperature which could be reached was $40^\circ$. Most of the films became fluid at temperatures above $50^\circ$. Therefore a simpler method was used with the higher melting materials. A film was spread on one liter of test solution in a pyrex dish and dusted lightly with talc to visualize it. When the film was the solid-condensed variety, such as stearic acid on alkaline earth solutions or distearin on water, the talc particles were completely immobilized. The solution was then heated until the film became fluid. This typically occurred over a $5-10^\circ$ temperature range and is analogous to taking the melting point of a bulk phase material.

Fatty acids

Caprylic, capric, lauric and myristic acid were obtained from Applied Science Laboratories. They were 99.8% pure and were used
without further purification. Palmitic acid and stearic acid were purified in this laboratory and had an Iodine number of .01. Their melting points agreed with literature values to within a degree. 

\[(PA = 63-64^\circ, SA = 67-68^\circ)\]

Oleic and elaidic acid were also purified previously in this laboratory. Both had an Iodine number of 89.5. Oleic acid was a colorless liquid at room temperature. Elaidic acid was a white powder melting at 50\(^\circ\). Methyl esters were prepared from palmitic, stearic, oleic and elaidic acid and examined by gas chromatography. No impurities could be detected at the 99 per cent level.

1,3-dipalmitin and 1,3-distearin were obtained from Proctor and Gamble Company and recrystallized from ethanol before use. DP melted at 68\(^\circ\) and DS at 76\(^\circ\). DL-\(\alpha\beta\)-dihexadecyl glycerol ether (m.p. 55\(^\circ\)) and DL-\(\alpha\beta\)-dioctadecyl glycerol ether (m.p. 65\(^\circ\)) were obtained from Dr. Morris Kates and used without further purification.

Cholesterol was obtained from Pfanstiehl Chemical Company and recrystallized twice from ethanol before use.

Water was glass distilled into polyethylene containers. It had a specific conductance of \(1.4 \times 10^{-6} \Omega\) and did not stiffen a stearic acid film at pH 9.0.

Commercially available salts were used. They were Mallinckrodt MgCl\(_2\)·6H\(_2\)O, CaCl\(_2\)·2H\(_2\)O, SrCl\(_2\)·6H\(_2\)O, BaCl\(_2\)·2H\(_2\)O, NaCl, NaOH and HCl, of analytical reagent grade.

Test materials were spread from acid washed hexane distilled between 68-70\(^\circ\). Neither material left a detectable amount of surface
active material if spread in pure form. The pH of subphase solutions was adjusted with 0.1 N HCl and NaOH. A Corning Model 12 pH meter was used to monitor pH before and after each run.

Phosphatides

Phosphatidyl choline was prepared from egg lecithin by the method of Rhodes and Lea (72). The yolks of twelve eggs were separated from the whites and washed with distilled water. They were homogenized in 500 ml acetone and filtered at 4°C. This was repeated three times. The residue was extracted with 300 ml of chloroform:methanol 1:1 v/v in a blender for 15 minutes. The combined extracts were evaporated under a water vacuum and dissolved in 100 ml of petroleum ether, then precipitated with 800 ml acetone, centrifuged and the supernatant decanted and discarded. This was repeated twice. The precipitate was dissolved in 100 ml of petroleum ether and allowed to set overnight at 4°C, then centrifuged to remove the crystallized sphingomyelin. The resulting solution contained approximately 0.1g per ml of crude egg phospholipids.

Twenty ml of the solution was taken to dryness in vacuo and the PL taken up in 20 ml of chloroform:methanol 1:1. (Mallinnchrodt A.R. grade) This was placed on 40 g of Woelm basic aluminum oxide in a column 2 cm in diameter and 30 cm long, and eluted with the same solvent. The lecithin fraction eluted in the first 150 ml.

The crude lecithin was further purified on Unisil (Clarkson Chemical Co.) Approximately 30 mg of PC phosphorous was taken up in 5 ml of chloroform:methanol (68:32, v/v) placed on 24 g of Unisil in the same column and eluted with the same solvent. PC eluted with 100-500 ml of solvent.
The column elution and purity of the preparation were followed with thin layer chromatography on Silica Gel G, using chloroform:methanol:water 68:25:4 v/v as the solvent system.

The PC fraction showed no impurities on TLC and was used in the experiments.

Phosphatidic acid was prepared from PC by a modification of the Kates procedure (73). Ten grams of cabbage leaves were homogenized for 2 minutes in 100 ml water and filtered through cheese-cloth. The filtrate was extracted twice with ether to remove the greater portion of cabbage lipids. 30 ml of the aqueous phase containing phospholipase D was combined with 300 mg of pure PC, 30 ml of .1M acetate buffer, pH 5.6, and 10 ml of 1 M CaCl₂. This mixture was allowed to stand at room temperature for two hours, then extracted twice with ether. The ether extracts were combined and dried over Na₂SO₄, the ether then being removed in vacuo.

The crude PA was taken up in 5 ml of chloroform:methanol (98:2), placed on a 24 g Unisil column and eluted with the same solvent. Pure phosphatidic acid eluted in 100-150 ml of solvent. These fractions were combined and taken to dryness. The PA was dissolved in 10 ml ether, then precipitated by adding 50 ml acetone, followed by centrifugation. The PA collected in the bottom of the centrifuge tube as a clear, gelled material. The supernatant was discarded, 10 ml of distilled water were added and the PA stirred with a glass rod. It quickly turned to a white, waxy solid which could be removed, dried and stored. This gave a single spot on TLC, moving near the solvent front in chloroform:methanol:water 68:25:4.
Phosphorous was determined by the method of Lowry et al (78). The following reagents were prepared:

**Ashing mixture.** 16.25 ml of 70% HClO₄ and 69.8 ml of H₂SO₄ were placed in a 250 ml flask and diluted to volume.

**Color developer.** 2.5 g of ammonium molybdate and 13.61 g of sodium acetate.3 H₂O were placed in a one liter flask and diluted to volume. This was kept at 4°. Just before use, one gram of ascorbic acid was added to 100 ml of the solution and thoroughly dissolved.

**Standard.** A stock solution was made up containing 200 ug phosphorous per ml. (173 mg of Na₂HPO₄ made to 100 ml.) The working solution was a 1:20 dilution of the above, containing 10 ug P/ml.

**Procedure.** .1, 15 and 1.0 ml of the standard working solution were added to 20 ml test tubes which had been carefully cleaned with Ivory soap. (This does not contain any phosphate.) Samples were added to each tube. A blank was prepared containing only the ashing mixture. The tubes were heated in a sand bath for two hours at 160°, until the char disappeared, then cooled. Ten ml of the color developer were added and the tubes incubated at 37° for 2 hours, then read in a Beckman DU spectrophotometer using a wavelength of 820 mu.

Phosphatidyl serine was obtained from International Chemical and Nuclear Corporation. This material showed two equally dense spots on TLC, one of which corresponded to phosphatidyl ethanolamine. The other spot was assumed to be phosphatidyl serine.

The PS was purified by column chromatography on 10 g of Unisil, using chloroform:methanol (1:1) as a solvent. The PE came off with some PS in the first 40 ml, and fractions containing pure PS were collected between 60 and 100 ml.
DISCUSSION OF RESULTS

Action of calcium on fatty acid films

Several explanations of the effect of divalent ions on fatty acid monolayers have appeared in the literature with little or no experimental evidence to support them. Abood has suggested that part of the effect may be due to the destruction of charge on the fatty acid ion when the divalent ion interacts with it (55), (56). This would reduce repulsion between the molecules and allow greater interaction. This possibility has also been mentioned by Kavanau (74).

Harkins (75) and Archer (76) have suggested that the alkaline earth ion may be forming a copolymeric lattice structure with the fatty acid film. Calcium has a coordination number of eight in most of its crystals. For instance, in anhydrous calcium soaps each calcium is surrounded by eight equivalent oxygens. (The carboxyl group oxygens are equivalent due to resonance of the charge.) Therefore a single calcium could be coordinating with four fatty acid oxygens, the other four sites being filled by water. This would produce the lattice structure diagrammed in Fig. 5, having the correct stoichiometric ratio of two fatty acids per calcium. In this case, the main contribution to film strength are the copolymeric soap lattice bonds.

The most generally accepted explanation is that a single calcium interacts with two fatty acid ions to produce a calcium distearate molecule. (This will be called a disoap.) This has been proposed by Harkins (75), Abood (55) (56), Shulman (77), Spink and Sanders (78) and Kavanau (74).
Figure 5. Copolymeric Soap Lattice Structure
Formation of a disoap might increase film viscosity in two ways:

1) The molecule has a longer effective chain length. The effect of increased chain length can be seen in numerous instances in the bulk phase. For instance, N-hexadecane is liquid at room temperature \((MP = 20^\circ)\) whereas increasing the chain length by two carbons to N-octadecane produces a solid at room temperature \((MP = 29^\circ)\).

2) Rotational freedom is lost. The oblong shape of a calcium distearate molecule would tend to prevent free rotation.

Both of these result in increased van der Waal's interactions in the film molecules and these forces would be the main contributors to increased film viscosity.

In the present study an attempt was made to prove the latter case, that simple soaps were being formed. To do this, a comparison was made between the surface properties of fatty acid films on solutions of alkaline earth ions and properties of simple salt analogs in which two long chain hydrocarbons were covalently bound together through a hydrophilic head. If simple salts were being formed, surface properties of the two types of films should be similar.

The analog compounds chosen were 1,3-diglycerides and 1,2-glycerol diethers. Molecular models showed that packing possibilities of the hydrocarbon chains in these compounds are similar to the packing which would result if two fatty acids are bound through a calcium ion. (Fig. 6, 7, 8) Experimental evidence for this will be presented later.

First let us consider the suggestion that charge destruction may contribute to film viscosity. Fig. 9 shows surface viscosities of SA on water, SA on .02 N HCl, SA on 1 M NaCl, and octadecanol on water.
Figure 6. Calcium Stearate Disoap
Figure 7. 1, 3 - Distearin
Figure 8. 1, 2 - Glycerol Distearyl Ether
Figure 9. Surface Viscosity/Surface Pressure
Since removing the charge by lowering the pH, by interaction with a monovalent ion at very high concentration, or by replacing it with an uncharged functional group does not greatly affect film viscosity, it is concluded that charge destruction plays a minor role, if any.

Fig. 10 shows force/area curves for SA on water at high and low pH values. pH alone does not seem to affect the curve. The curve shows the existence of two phases, one of which has an area/molecule of 25 A\(^2\) at zero pressure, the other having an area of 21 A\(^2\) when extrapolated to zero pressure. The 21 A\(^2\) figure is due to the upright packing of the hydrocarbon chains, remaining constant with varying chain length. The packing cross section is quite similar to the bulk phase value, which is 18 A\(^2\).

The 25 A\(^2\) figure has been attributed by Singleton and others to the cross sectional area of the hydrophilic head (79). The early experiments with fatty acid films suggested that on distilled water the film seemed to be quite a bit more condensed than on dilute HCl solutions (80). Singleton proposed that "When the film is spread over distilled water, the cross section on the chain gives the effective area because of the different depths to which alternate molecules enter into the water. When the pH of the water is decreased, as in the case of 0.01 N hydrochloric acid solution, the attraction of the carboxyl group for the water decreases and forces these groups to the same level. The area, 25 x 10\(^{-16}\) sq. cm. per molecule, therefore, corresponds to the cross section of the polar (COOH) group." (79)

Since the main effect of calcium on force/area curves is the loss of the liquid-condensed portion of the curves, an understanding
Figure 10. Force/Area Curves for Stearic Acid on Water
of this effect would help visualize what may be occurring. Several facts do not agree with Singleton's theory:

1) The data which he used to support it has since been shown to be in error. The early workers did not realize the profound effect small amounts of polyvalent ions could have on monolayer properties. The condensed curve found on distilled water was actually due to impurities in the water such as copper or calcium which interacted with the film. These did not interact at low pH ranges. Actually there is no difference in force/area curves at high and low pH if the water is free of ions (81). The present data confirms this.

2) Hydrogen bonding cannot occur between fatty acid molecules in monolayers. This was proved by Alexander (82). If the carboxyl groups are large enough to show steric hindrance, as Singleton suggested, they would probably be close enough to form hydrogen bonds.

3) Long chain alcohols also have the liquid-condensed portion of the curve. (Fig. 11) The -OH group certainly is not large enough to show steric hindrance with neighboring molecules.

An alternative theory is as follows:

If the two areas are compared visually, it can be seen that only a small difference in actual area is present.
Figure 11. Force/Area Curve for Octadecanol on Water
Obviously the hydrocarbon chains are almost as tightly packed at 25 A^2 as at 21 A^2. There are three manners in which fatty acids may pack in the bulk phase, called A, B, and C packing. In A packing, the chains are perpendicular to the smectic phase, in B packing they are tilted in one direction and in C packing they are tilted in a second, additional direction. (Fig. 12, 13, 14) In the monolayer it is still uncertain which type of packing is present, but most likely during compression there is a mixture which eventually resolves to a single type at greatest compression.

To change from one type of packing to another molecules must slide up and down each other. I suggest that the 25 A^2 figure is probably a result of this motion, the increased area being due to steric hindrance of hydrogens coming into opposition. Thus, any fixation of the chains with respect to one another would tend to allow tighter packing, as when calcium condenses the film or in solid-condensed films such as di- and triglycerides. This in turn would result in loss of the liquid-condensed portion of the curve.

This suggestion is supported by a calculation of the area occupied by hydrocarbon chains when the hydrogens are alternating and when they are opposed. Using a van der Waal's radius of 1.3Å for hydrogen, the value for hydrogen in methyl groups, the distances between carbon chains in the case of alternating hydrogens are 4.4 and 4.55Å. This gives an area per molecule of 4.4 x 4.55 = 20.0 Å^2. When the hydrogens are opposed, the distances are 4.8 and 4.9 Å, giving an area per molecule of 23.6 Å^2. In the present study, the experimental value (∆A^2) was 4.1 Å^2, (Fig. 10) and Shanes found a value of 3.7 Å^2 (54).
Figure 12. Type A Packing
Figure 13. Type B Packing
Figure 14. Type C Packing
Both figures compare favorably with the calculated value.

The affect of calcium on the liquid-condensed portion of the SA force/area curve can be seen in Fig. 15. The 25 Å² area has disappeared and the film is entirely condensed. If talc is sprinkled on the film after being spread but before compression, large, solid islands can be observed. The quickly rising portion of the curve is simply a result of these islands being crushed together.

The smallest concentration of calcium which can affect the curve is approximately $10^{-6}$ M. (Fig. 16) In the volumes used in the trough, this is equivalent to about 15 calcium ions per fatty acid. If the film could remove every ion from solution the ideal figure would be two fatty acids per calcium ion, but even a 1:1 ratio ($10^{-7}$ M) has no affect on the film.

Spink and Sanders (78) showed that pH has a definite affect on calcium interaction with films. (Fig. 17) As pH decreases, hydrogen ion begins to compete with calcium for anionic sites and the liquid-condensed portion of the curve returns.

This effect was used to study the relative strength of binding of the four alkaline earth ions to the film. (Figs. 18, 19, 20) The order at the three pH values are:

<table>
<thead>
<tr>
<th>pH</th>
<th>Ca</th>
<th>Mg</th>
<th>Sr</th>
<th>Ba</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>Ca</td>
<td>Ba</td>
<td>Sr</td>
<td>Mg</td>
</tr>
<tr>
<td>6.0</td>
<td>Ba</td>
<td>Sr</td>
<td>Ca</td>
<td>Mg</td>
</tr>
</tbody>
</table>

These results will be discussed later with reference to a second method for determining this order.
Figure 15. Force/Area Curve for Stearic Acid on 0.2mM Calcium, pH 9.0
Figure 16. Force/Area Curves for Stearic Acid on Calcium Solutions of Varying Concentrations
Figure 17. Force/Area Curves for Stearic Acid on Calcium Solutions of Varying pH
Figure 18. Force/Area Curves for Stearic Acid on Alkaline Earth Ions, pH 9.0

SA on .2 mM ions, pH 9.0
Figure 19. Force/Area Curves for Stearic Acid on Alkaline Earth Ions, pH 7.5
Figure 20. Force/Area Curves for Stearic Acid on Alkaline Earth Ions, pH 6.0

SA on .2mM ions, pH 6.0
Fig. 21 shows the area/chain for distearin and distearyl ether, compared to SA on calcium solutions. As was indicated by the molecular models, the packing of the chains in the three instances is very similar, if not identical, suggesting that the analogy is reasonable. It is interesting to note that the liquid-condensed phase has disappeared from the diglyceride and diether curves.

Fig. 22 compares the effects of adding double bonds to the chains. Oleic acid forms a liquid expanded film, as does elaidic acid. In oleic acid this is mainly due to the bond in the chain which prohibits any sort of regular packing. With elaidic acid there is no real bend in the chain, but nonetheless the film is of the liquid expanded variety, although more condensed. A $\pi$-bond orbital takes up somewhat more room than s-orbital bonding. It may be that this discontinuity in the chain prevents the very close packing necessary to form a condensed film.

Calcium seems to have no affect on the force/area curve for oleic acid. (Fig. 23) Evidently rigid spatial requirements must be fulfilled before divalent ions can produce the typical condensation seen with saturated fatty acid films. The result with elaidic acid is surprising. Calcium actually seems to weaken the film, which has a much lower collapse pressure (Fig. 24). No other effect is apparent. Possibly calcium binds to the elaidic acid film in such a manner as to disallow the chain packing necessary to form a more coherent film.

Surface viscosity studies generally support the results of the force/area curves, in that films which are of the solid-condensed variety also have high surface viscosities. Palmitic and stearic acid
Figure 21. Force/Area Curves for Disoap Analogs
Figure 22. Force/Area Curves for Oleic, Elaidic and Stearic Acid
Figure 23. Force/Area Curve for Oleic Acid on Calcium Solutions
Figure 24. Force/Area Curves for Elaidic Acid on Calcium Solutions
on water have a surface viscosity of $3-4 \times 10^{-4}$ surface poises at low surface pressures. This is in good agreement with the literature. (Table 11) The PA and SA film on .2 mM calcium above pH 6 have an immeasurable surface viscosity. The films become so rigid that no flow occurs through the slit. Capric, lauric and myristic do not form stable films, either on water or on calcium solutions.

If formation of a simple soap is responsible for this increment in film strength, the simple soap analogs should also form high viscosity films. Fig.9 shows that this is generally true, although the viscosities are still not nearly so high as these of saturated fatty acids on calcium.

Finally, calcium has no affect on the surface viscosity of oleic and elaidic acids over the entire pH and pressure range, again demonstrating the rigid spatial requirements for the stiffening of monolayers by divalent ions. (Table 11) Since possibly the size of the ion was important in forming the rigid film, barium was also tried, again without effect.
Table 11. Surface Viscosity of Monolayers of Fatty Acids and Fatty Acid Derivatives

<table>
<thead>
<tr>
<th>Material</th>
<th>Conditions</th>
<th>$T$ (°C)</th>
<th>$S$ (cm)</th>
<th>$\pi$ (dynes/cm)</th>
<th>$\eta_0$ (mPa·s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic Acid</td>
<td>H$_2$O pH 7.0</td>
<td>28°</td>
<td>.1</td>
<td>5</td>
<td>$4.3 \pm 0.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>.2mM Ca$^{2+}$ pH 9.0</td>
<td>28°</td>
<td>.1</td>
<td>5</td>
<td>$\infty$</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>H$_2$O pH 7.0</td>
<td>28°</td>
<td>.1</td>
<td>5</td>
<td>$5.0 \pm 0.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>.2mM Ca$^{2+}$ pH 9.0</td>
<td>28°</td>
<td>.1</td>
<td>5</td>
<td>$\infty$</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>H$_2$O pH 3.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$3.0 \pm 0.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>.2mM Ca$^{2+}$ pH 3.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$3.7 \pm 0.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>1.0 M Na$^+$ pH 7.0</td>
<td>28°</td>
<td>.1</td>
<td>5</td>
<td>$5.5 \pm 1.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>Capric, lauric Myristic Acid</td>
<td>No stable film.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>H$_2$O pH 7.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$2.6 \pm 0.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>2 mM Ca$^{2+}$ pH 7.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$2.9 \pm 0.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>20 mM Ca$^{2+}$ pH 7.0</td>
<td>29°</td>
<td>.1</td>
<td>5</td>
<td>$1.7 \pm 0.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>2mM Ba$^{2+}$ pH 7.0</td>
<td>29°</td>
<td>.1</td>
<td>5</td>
<td>$2.0 \pm 0.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>2mM Ca$^{2+}$ pH 9.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$2.5 \pm 0.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Elaidic Acid</td>
<td>2mM Ca$^{2+}$ pH 7.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$2.6 \pm 0.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Elaidic Acid</td>
<td>H$_2$O pH 7.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$2.5 \pm 0.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Elaidic Acid</td>
<td>2mM Ca$^{4+}$ pH 9.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$2.4 \pm 0.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Octadecanol</td>
<td>H$_2$O pH 7.0</td>
<td>28°</td>
<td>.1</td>
<td>5</td>
<td>$13.8 \pm 1.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>Dipalmitin</td>
<td>H$_2$O pH 7.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$1.6 \pm 0.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>Distearin</td>
<td>H$_2$O pH 7.0</td>
<td>28°</td>
<td>.1</td>
<td>5</td>
<td>$4.5 \pm 1.3 \times 10^{-2}$</td>
</tr>
<tr>
<td>Octadecyl glycerol diether</td>
<td>H$_2$O pH 7.0</td>
<td>26°</td>
<td>.1</td>
<td>5</td>
<td>$9 \pm 3 \times 10^{-1}$</td>
</tr>
</tbody>
</table>
From the results with saturated fatty acids and simple soap analogs, it seemed that the formation of a simple soap could explain at least some of the effects of divalent ions on fatty acid films. Monolayers of diglycerides and diethers were of the solid-condensed type and were much more viscous than monolayers of the parent fatty acids.

For a definitive experiment, a check of "surface melting points" was proposed. The reasoning was as follows:

The choice between alternatives was still one of deciding between increased van der Waal's forces and a copolymeric lattice structure. In the bulk phase, the ionic lattice structure is a much stronger bonding force. This is reflected in the difference between melting points of compounds in which van der Waal's forces are predominant and compounds in which an ionic lattice is present.

For instance, doubling the effective chain length of N-octadecane in various ways produces a 40° increase in melting point, whereas adding a lattice structure to it, as in alkali metal or alkaline earth soaps of fatty acids, increases the melting point by about 130°. (See Table 12.) The effect of the ionic lattice has also been noted in phospholipid bulk phase by Byrne (83). Phospholipids generally melt at 200° or more, whereas their fatty acid moieties melt around 20°.

Therefore, if fatty acid films form a disoap on calcium solutions, the temperature at which they become fluid should be similar to the temperature at which monolayers of the disoap analogs liquify. These results are shown in Table 13.
Table 12. Bulk Phase Transition Temperatures

<table>
<thead>
<tr>
<th>Compound</th>
<th>M.P.</th>
<th>Compound</th>
<th>M.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 18 Hydrocarbon</td>
<td>28°</td>
<td>C 16 Hydrocarbon</td>
<td>18°</td>
</tr>
<tr>
<td>C 36 Hydrocarbon</td>
<td>76°</td>
<td>C 32 Hydrocarbon</td>
<td>70°</td>
</tr>
<tr>
<td>Distearin</td>
<td>79°</td>
<td>Dipalmitin</td>
<td>70°</td>
</tr>
<tr>
<td>Distearyl ether</td>
<td>65°</td>
<td>Dipalmityl ether</td>
<td>60°</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>69°</td>
<td>Palmitic acid</td>
<td>64°</td>
</tr>
<tr>
<td>Calcium distearate</td>
<td>≈ 180°</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium dipalmitate</td>
<td>≈ 180°</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium dilaurate</td>
<td>≈ 180°</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 13. Monolayer Phase Transition Temperatures

<table>
<thead>
<tr>
<th>Compound</th>
<th>M.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipalmitin</td>
<td>25-30°</td>
</tr>
<tr>
<td>Distearin</td>
<td>50-55°</td>
</tr>
<tr>
<td>Dipalmityl ether</td>
<td>67-69°</td>
</tr>
<tr>
<td>Distearyl ether</td>
<td>70-75°</td>
</tr>
<tr>
<td>Palmitic acid on .2mM Ca, pH 9.0</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td>Stearic acid on .2mM Ca, pH 9.0</td>
<td>&gt; 90°</td>
</tr>
</tbody>
</table>
The analog compounds all melt at temperatures equal to or less than their bulk phase melting points. Fig. 25 demonstrates this in DP and DS in terms of a measured viscosity change. However, SA and PA on calcium solutions at pH 9.0 do not melt, even when the water is nearly boiling. If a stearic acid film is spread on calcium at 90° and dusted with talc, solid islands can be seen to form immediately.

Melting points of SA films on the four alkaline earth ions are shown in Fig. 26. Taking the melting point as an indication of binding strength of the ions, calcium forms the strongest film at pH 8 and 9, while magnesium forms the weakest film. Barium and strontium are relatively unaffected by pH, and barium forms the strongest film at pH 6 and 7. These findings are in general agreement with the earlier pH results, and are compared in Table 14 below:

Table 14. Binding Strength of Divalent Ions

| Melting point: | pH 9.0 | Ca > Ba > Sr > Mg |
|               | pH 7.0 | Ba > Ca > Sr > Mg |
|               | pH 6.0 | Ba > Sr > Ca > Mg |

| Hydrogen ion competition: | pH 9.0 | Ca > Mg > Sr > Ba |
|                          | pH 7.5 | Ca > Ba > Sr > Mg |
|                          | pH 6.0 | Ba > Sr > Ca > Mg |

From the high melting point of fatty acid films on calcium solutions at pH 8.0 and 9.0, relative to the simple soap analogs, it is concluded that calcium is binding in such a manner as to form a copolymeric lattice structure. This is diagrammed in figure 5, and a photograph of molecular
Figure 25. Viscosity of Dipalmitin and Distearin
Figure 26. Surface Phase Transition Temperatures for Stearic Acid on Alkaline Earth Ions
models of this structure is seen in Fig. 27. As pH decreases, less
and less lattice structure is possible, due to hydrogen ion competing
with calcium for binding sites. At pH 5, no lattice can form. Bagg
demonstrated that at this pH about 50% of the stearic acid is ionized
(84). This is similar to the figure arrived at by diluting the stearic
acid with octadecanol, where the mole fraction of stearic acid must
be greater than 0.5 for calcium to have an effect on film viscosity.

Table 15. Effect of Octadecanol on SA Phase

<table>
<thead>
<tr>
<th>Mole Fraction SA</th>
<th>Film Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>.1</td>
<td>liquid</td>
</tr>
<tr>
<td>.2</td>
<td>liquid</td>
</tr>
<tr>
<td>.3</td>
<td>liquid</td>
</tr>
<tr>
<td>.4</td>
<td>liquid</td>
</tr>
<tr>
<td>.5</td>
<td>liquid</td>
</tr>
<tr>
<td>.6</td>
<td>plastic</td>
</tr>
<tr>
<td>.7</td>
<td>rigid</td>
</tr>
<tr>
<td>.8</td>
<td>rigid</td>
</tr>
<tr>
<td>.9</td>
<td>rigid</td>
</tr>
<tr>
<td>1.0</td>
<td>rigid</td>
</tr>
</tbody>
</table>

The difference between the various alkaline earth ions in terms of
binding strength may be explained by the geometry of the lattice structure.
In chelation studies of the alkaline earths, chelate stability increases
Figure 27. Model of Lattice Structure
linearly with decreasing ionic radius: \( \text{Mg} > \text{Ca} > \text{Sr} > \text{Ba} \) (85). This is mainly due to the greater charge density of the smaller ion having an enhanced ability to polarize to anion, producing a stronger bond. However, in EDTA chelates, calcium has an anomalously high association constant which has been attributed mainly to a favorable geometry of the sexadentate ring which forms. Fig. 5 shows the four ions superimposed on the probable carboxyl group configuration of a SA monolayer. The magnesium ion may be somewhat too small, and strontium and barium somewhat too large for an ideal fit.

Phospholipid films

The main objective in the phospholipid film work was to determine whether the presence of calcium altered the surface properties, in particular surface viscosity, of phospholipid monolayers under various conditions.

Egg phosphatidyl choline forms liquid-expanded monolayers. (Fig. 28) This could be predicted from a knowledge of its fatty acid composition, which includes a large fraction of unsaturated acids. If egg lecithin is hydrogenated the force/area curve is more condensed, and the area/molecule at collapse pressure corresponds to that of two saturated hydrocarbon chains. (Fig. 28)

With the unsaturated material, the first surface pressure changes occur at an area of approximately 150-200 \( \text{A}^2/\text{molecule} \) and at the collapse pressure the area is 65-70 \( \text{A}^2/\text{molecule} \).

Garvin and Karnovsky have shown that both the phosphate and choline group are charged between pH 1 and 10 (86). Therefore pH should have little affect on the force/area curve and Fig. 29 shows that this is the case.
Figure 28. Force/Area Curve for Phosphatidyl Choline on Water, pH 7.0
Figure 29. Force/Area Curve for Phosphatidyl Choline on Water, Varying pH
Stoekenius found that bulk phase phospholipid under certain conditions of temperature and water content could undergo a change of phase from a lamellar to a hexagonal form (22). Temperature was varied in the present system to see if such a change of phase might also be demonstrated in monolayers of phosphatidyl choline. However, as Fig. 28 shows, temperature has little affect on the shape of the curve between 20° and 45°. Phosphatidyl choline spread on 2 and 20 mM calcium solutions at pH 7.0 behaved no differently than when it was spread on water (Fig. 30). This agrees with the findings of Rojas (60) who spread PC, PS and PE on water and on calcium solutions. Only PS showed a calcium effect, its force/area curve becoming somewhat more condensed.

Since phospholipid is associated with large amounts of cholesterol in the cell membrane, the possibility existed that certain changes of phase induced either by ions or temperature or a combination of these variables were dependent upon the presence of cholesterol. Cholesterol by itself forms a very liquid monolayer with a condensed type of force/area curve. (Fig. 31) It has a limiting area of 39 Å²/molecule and shows almost no compressibility.

PC and cholesterol in a 1:1 mole ratio mixture give a somewhat more condensed force/area curve than one might expect, as has been mentioned previously (32). Fig. 33 shows the affect of temperature on the PC:cholesterol mixture. The curves remain remarkably constant over the entire range of temperatures.

The presence of cholesterol does not seem to enhance any action which calcium might have and curves with and without divalent ions present superimpose each other. (Fig. 34)
Figure 30. Force/Area Curve for Phosphatidyl Choline on Calcium Solutions
Figure 31. Force/Area Curve for Cholesterol on Water
Figure 32. Force/Area Curve for PC:Cholesterol on Water
Figure 33. Force/Area Curve for PC:Cholesterol on Water, Varying Temperature
Figure 34. Force/Area Curve for PC:Cholesterol on Calcium Solutions
Surface viscosity

Table 16 shows the surface viscosity of PC on water and on 2 mM calcium at different temperatures. Calcium ions have no affect on the surface viscosity of PC in the temperature range employed, even though Koketsu showed that it is definitely binding in a 2:1 PC:calcium ratio (58).

The surface viscosity of cholesterol is very low. When it is mixed in a 1:1 mole ratio with PC, no change is observed in the surface viscosity of the mixture, indicating that even though cholesterol tends to condense the phospholipid monolayer, this effect does not change the phase of the monolayer. The presence of calcium in the subphase has no affect on the surface viscosity of the mixed PC:cholesterol monolayer.

When calcium ions bind to a neutral phospholipid film, the film gains a positive charge. The negative results up to this point possibly could be explained by this charge. Therefore the surface viscosity of phosphatidic acid produced from egg lecithin by the action of phospholipase D was measured on water and calcium solutions. Again, calcium had no effect. A second possibility was that the unsaturated nature of the hydrocarbon chains was keeping the anionic groups too far apart for the calcium ion to interact effectively, similar to the disruption of the calcium effect in oleic acid monolayers by the cis 9-10 double bond. Therefore a sample of pure hydrogenated egg phosphatidylocholine was spread on water and on calcium solutions. Surprisingly, the surface viscosity of this material was similar to that of unsaturated PC. From the results with diglycerides and diethers it might have been
### Table 16. Surface Viscosity of Phosphatides

<table>
<thead>
<tr>
<th>Material</th>
<th>Conditions</th>
<th>I</th>
<th>Slit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl choline</td>
<td>H₂O pH 7.0</td>
<td>26°</td>
<td>.1</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>2 mM Ca⁺⁺ pH7.0</td>
<td>26° → 39°</td>
<td>.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>H₂O pH 7.0</td>
<td>28°</td>
<td>.1</td>
</tr>
<tr>
<td>PC : Cholesterol</td>
<td>H₂O pH 7.0</td>
<td>24°</td>
<td>.1</td>
</tr>
<tr>
<td>PC : Cholesterol</td>
<td>2mM Ca⁺⁺ pH 7.0</td>
<td>24°</td>
<td>.1</td>
</tr>
<tr>
<td>Phosphatidic Acid</td>
<td>H₂O pH 7.0</td>
<td>28°</td>
<td>.1</td>
</tr>
<tr>
<td>Phosphatidic Acid</td>
<td>2mM Ca⁺⁺ pH 7.0</td>
<td>28°</td>
<td>.1</td>
</tr>
<tr>
<td>Hydrolecithin</td>
<td>H₂O pH 7.0</td>
<td>26°</td>
<td>.1</td>
</tr>
<tr>
<td>Hydrolecithin</td>
<td>2 mM Ca⁺⁺ pH 7.0</td>
<td>26°</td>
<td>.1</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>H₂O pH 7.0</td>
<td>28°</td>
<td>.1</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>2mM Ca⁺⁺ pH 7.0</td>
<td>28°</td>
<td>.1</td>
</tr>
<tr>
<td>PC</td>
<td>H₂O pH 3.2</td>
<td>26°</td>
<td>.1</td>
</tr>
<tr>
<td>PC</td>
<td>H₂O pH 7.2</td>
<td>26°</td>
<td>.1</td>
</tr>
<tr>
<td>PC</td>
<td>H₂O pH 9.0</td>
<td>26°</td>
<td>.1</td>
</tr>
</tbody>
</table>
expected that saturated lecithins would also have a high intrinsic surface viscosity. Again, the presence of calcium in the subphase had no affect on the viscosity of the monolayer.

Since Rojas and Tobias had reported that only phosphatidyl serine showed a calcium effect either with force/area curves (which became somewhat more condensed) or with their millipore filter membrane model, it was decided to repeat the above experiments with PS. Fig. 35 shows force/area curves of PS on water and on calcium solutions. A slight condensing effect of calcium was noted, similar to that reported by Rojas. However, calcium had no affect on the surface viscosity of PS films at pH 7.0 and 9.0. (Table 16)

An attempt was made to repeat the Rosano experiment in hopes of measuring surface viscosity changes in the duplex film. Unfortunately, using egg phosphatidyl choline and employing their conditions and numerous variations of them, no duplex film was ever seen to form. Evidently their conditions, which were empirically derived for phosphatidyl ethanolamine of doubtful purity, are not satisfactory for work with egg PC.

Fig. 36 shows scale drawings of the geometry of PL phosphate groups in relation to the area occupied by the phospholipid molecule. Calcium ions are shown, also drawn to scale. It can immediately be seen that no lattice structure can occur as it evidently does in fatty acid films. At most, only 2:1 complexes of phospholipid to calcium can form.
Figure 35. Force/Area Curve for Phosphatidyl Serine on Calcium Solutions
Figure 36. Calcium Interaction with Phosphate Groups
SUMMARY AND CONCLUSIONS

Saturated fatty acids of sixteen carbons or more form solid films on solutions of alkaline earth ions at pH 8-9. As pH is decreased, the films become weaker, until at pH 5 the films are fluid. Oleic and elaidic acid do not form solid films on calcium or barium solutions in the pH range studied.

Reducing the charge on a fatty acid film by lowering the pH, by interaction with a high concentration of monovalent ion or by replacing the charged group with an uncharged group does not appreciably alter surface viscosity.

The disoap analogs, dipalmitin, distearin, glycerol dipalmityl ether and glycerol distearyl ether form highly viscous films of the solid-condensed variety. These have a surface phase transition temperature equal to or less than the bulk phase material. (30-65°). Palmitic and stearic acid films on calcium solutions at pH 8-9, however, have surface phase transition temperatures greater than 90°.

The surface viscosity of phosphatidyl choline, phosphatidyl serine and phosphatidic acid is unaffected by the presence of calcium. The presence of cholesterol, although having a condensing effect on phosphatidyl choline films, does not alter the surface viscosity or enhance any action which calcium may have. Temperature and pH have little effect on these films throughout the range studied.

From the very high phase transition temperatures relative to those of the disoap analogs, it is concluded that the increment in surface viscosity of saturated fatty acid films spread on solutions
of alkaline earth ions is probably due to formation of a copolymeric soap lattice in the plane of the monolayer, rather than production of disoap molecules. Formation of the lattice is strongly dependent on precise spatial relationships and pH.

Such a lattice does not form in phosphatidyl choline, phosphatidyl serine or phosphatidic acid films, probably owing mainly to lack of the rigid geometrical requirements which are present in the special case of saturated fatty acid films.

This finding can be extrapolated to the cell surface. In cell membranes, anionic phosphate groups are still farther apart than in phospholipid monolayers, due to the presence of large amounts of cholesterol. At most, only 2±1 phospholipid:calcium complexes could be formed, such as Koketsu has demonstrated in phospholipid monolayers. An understanding of changes induced in membrane structure by calcium must be based on this type of primary binding.

No rigid analogy should be drawn between the type of binding which occurs in fatty acid monolayers and the binding of calcium to cell membranes.
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