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LIPIDS

DISSERTATION
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By
Lester Irwin Burke, B.S.

The Ohio State University
1971

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Studies in Lipid Biochemistry. Dr. David Cornwell
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CHAPTER I
INTRODUCTION AND HISTORICAL REVIEW

Surface Properties of Lipids

Lipids and other compounds which possess both hydrophobic and hydrophilic groups can form insoluble monolayers at the air-water interface. The molecular structures of the classical compounds, acids and alcohols, studied by monolayer techniques consist of a polar group attached to a long nonpolar chain. The hydrophilic group is oriented in the aqueous phase while the hydrophobic group counterbalances this effect. The balance between the two groups determines the stability of the monolayer.

The molecular structure, the surface pressure, the type of subphase, and the temperature determine the surface properties of the molecule. If all of these factors are held constant except for molecular structure, then it is possible to determine the shape of the molecule and the number and relative position of different functional groups within the molecule from monolayer studies. The molecular structures of cholesterol, batyl and chimyl alcohol, ergosterol, estriol, and other compounds were
determined by monolayer techniques. The application of monolayer techniques for structural identification requires the comparison of surface properties of the compound whose structure is unknown with compounds of known structure. After the molecular structure of the compound is elucidated by monolayer techniques, the validity of the proposed structure must be reconfirmed by other methods.

Adam (1) compared the stability of monomolecular films of long chain hydrocarbons with different terminal groups. He found that unstable monolayers formed when weak polar groups were associated with long chain hydrocarbons. As the polarity of the compound increased, the stability of the monolayer improved until the water attracting properties of the polar group became sufficient to dissolve the molecule in the subphase (Table 1).

Molecular Structure

One of the earliest applications of monolayer techniques was the structural analysis of chimyl and batyl alcohol (2). (Formula I and II)
TABLE 1
EFFECTIVENESS OF FUNCTIONAL GROUPS IN PROVIDING
ATTRACTION TO WATER

<table>
<thead>
<tr>
<th>Very Weak (No film)</th>
<th>Weak (Unstable film)</th>
<th>Strong (Stable film with C16 Chain)</th>
<th>Very Strong (C16 Chains compounds dissolve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocarbon</td>
<td>-CH₂OCH₂</td>
<td>-CH₂OH</td>
<td>-SO₃⁻</td>
</tr>
<tr>
<td>-CH₂I</td>
<td>-C₆H₄OCH₃</td>
<td>-COOH</td>
<td>-OSO₃⁻</td>
</tr>
<tr>
<td>-CH₂Br</td>
<td>-COOCH₃</td>
<td>-CN</td>
<td>-NR₃⁺</td>
</tr>
<tr>
<td>-CH₂Cl</td>
<td></td>
<td>-CONH₂</td>
<td>-C₆H₄SO₄⁻</td>
</tr>
<tr>
<td>-NO₃</td>
<td></td>
<td>-CH=NOH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-C₆H₄OH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-CH₂COCH₃</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-NHCONH₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-NHCOCH₃</td>
<td></td>
</tr>
</tbody>
</table>

After Heilbron and Owens (3) isolated batyl alcohol, they postulated that the long chain hydrocarbon was located in the β-position of glycerol but they later questioned their structure. Knight (2) found the force-area isotherms of batyl and chimyl alcohols were similar to α-monopalmitin (Formula III). Since the data for β-monoglycerides were not available, n-octadecylmalonic acid (Formula IV) was used for comparison and it gave a larger area than either chimyl or batyl alcohol.
Therefore Knight concluded that chimyl and batyl alcohol were $\alpha$-glyceryl ethers rather than the $\beta$-glyceryl ethers which were first proposed. After Heilbronn et al. (4,5) synthesized both the $\alpha$- and $\beta$-glyceryl ethers, Adam (6) and Adam and Harding (7) concluded from monolayer studies on these synthetic glyceryl ethers that the $\alpha$-glyceryl ether was the naturally occurring structure. Since $\alpha$-monopalmitin and n-octadecylmalonic acid could be used as reference compounds, monolayers properties depend more on similarities in the position of the long chain hydrocarbon within the molecule than on the types of polar groups associated with the molecule.

In 1932 Bernal (8) challenged the structural formula of cholesterol proposed by Windaus and Wieland (Formula V and VI). From X-ray studies Bernal noticed that the cross section area occupied by the molecule perpendicular to its long axis was too small for the older formula and the
length of the molecule was longer than expected from the older formula. Adam (9) confirmed the X-ray data of Bernal when he determined the surface area of a condensed film of cholesterol was $40A^2$/molecule instead of $54A^2$/molecule which was the value expected from the stereochemical model of the Windaus and Wieland formula.

Adam et al. (10) used monolayer techniques to show that the molecular structure of estriol proposed by Butenandt and Stromer (11) was incorrect. (Formula VII, VIII, and IX)
Formula VII and VIII were structures of estriol proposed by Butenandt and Stromer (11). Formula IX was the structure of estriol proposed by Adam et al. (10).

Since estriol was too polar to form a stable monolayer, the triacetyl and diacetyl methyl ether derivatives were used. Both derivatives of estriol formed expanded films which form condensed films at low surface pressures (3-6 dynes/cm). Since the molecules easily assumed a flat position on the subphase (expanded film), Adam et al. (10) proposed that the phenolic hydroxyl was situated at the opposite end of the molecule from the two alcoholic groups. Furthermore, they concluded that the alcoholic groups were near one end of the molecule since the area of the condensed film was 32.5 Å²/molecule. Adam et al. rejected both molecular structures of Butenandt et al. since molecular models showed that condensed film for either structure was 70Å²/molecule. Butenandt (12) reinvestigated the molecular structure for estriol and showed that it was a four ringed structure which agreed with the surface film studies of Danielli et al. (13) (Formula X).

[Chemical structure diagram]

Present structure for estriol
Formula X
Thus surface chemistry was an essential tool in the early determination of molecular structure. Since cholesterol and estriol have similar surface properties, it was not surprising that their molecular structures were similar.

Danielli and Adam (14) used monolayer techniques to study the changes that occur in the molecular structure of ergosterol when it is irradiated to form calciferol or vitamin D. (Formula XI and XII)

![Ergosterol](Image)

**Ergosterol**
**Formula XI**

![Calciferol](Image)

**Calciferol**
**Vitamin D**
**Formula XII**

The changes in the location of the double bonds of ergosterol following irradiation were detected by monolayers studies on KMnO$_4$ subphase. These changes influenced
the accessibility of the double bonds to the subphase and affected the area. However Danielli and Adam could not predict the new location of the double bonds after irradiation.

Danielli and Adam (14) proposed that the hydroxyl group of ergosterol was located in the 3 position since the condensed film had a surface area of $37\text{Å}^2$ molecule. They constructed molecular models of ergosterol and found the calculated area/molecule of ergosterol from the molecular models agreed with the surface area measurements. When the hydroxyl group was moved to position 4, the tilt of the molecule increased the area of the condensed film to $42\text{Å}^2$ molecule. If the hydroxyl group was moved to position 1 or 2, a larger increase in area/molecule was noticed.

In a classical monolayer study Langmuir (15) showed that long chain saturated fatty acids have nearly the same force-area isotherms. The shape of the curve was independent of the length of the carbon chain provided there were more than 14 carbon atoms in the molecule. However, with very long chains the finer details of the curve were obliterated since the rigidness of the film did not respond readily to small changes in surface pressure. Since the area/molecule did not change as the length of the hydrocarbon chain varied, it was proposed that the molecules are oriented steeply to the surface and at the
same angle in all films. The surface area of long chain saturated fatty acids in condensed film is 20Å²/molecule which is almost the same as the cross section area in the bulk crystal determined by X-ray measurements (16). Electron diffraction studies on monolayers transferred to solids showed that the chains are oriented nearly vertically with respect to the surface, while optical measurements showed that the thickness of the film was that which was expected from fully extended chains (17).

The stability of fatty acid monolayers was studied recently in this laboratory (18, 19). Heikkila, Kwong, and Cornwell (18) examined the stability of different regions of the pressure-area isotherms of saturated fatty acids. Harkin and Boyd (20) and Stenhagen (21) reported that monolayers of saturated fatty acid undergo three phase transformations during compression at room temperature. The first phase (L₂) occurs at the initial area and is transformed to the LS phase when the film goes from an expanded to condensed film. The LS phase is changed to the S form before the collapse point is reached on the force-area isotherm. Heikkila et al. (18) have reported a phase transformation to the L₂ form at the equilibrium spreading pressure.

Fatty acids monolayers in the S-phase are related to the A crystalline structure while those in the LS phase have cross section areas similar to the B crystalline form.
Heikkila, Kwong, and Cornwell (18) proposed that the fatty acids at their equilibrium spreading pressures ($L_2$ phase) have cross section areas nearly identical to the C crystalline form.

In a similar study Heikkila, Deamer, and Cornwell (19) studied the reasons for the decrease in surface area at constant surface pressure (16 dynes/cm). They eliminated evaporation as the cause since the rate of film loss became greater with increasing pH. Collapse into the bulk phase did not explain the decrease in surface area since acids with equilibrium spreading pressures greater than 16 dynes/cm exhibited loss of area. Similarly acids with equilibrium spreading pressures just below 16 dynes/cm showed losses greater than could be explained solely on collapse. Porter et al. (22) proposed that autoxidation of unsaturated fatty acids followed by solution of short chain reaction products caused loss in surface area. However Heikkila et al. (19) showed loss of surface area with saturated and unsaturated fatty acids in either anaerobic or aerobic atmospheres. Furthermore their data were consistent with the hypothesis that fatty acid solution from the monolayer into the subphase was the mechanism for film loss (23). The loss was directly proportional to the solubility of saturated fatty acids and the number of double bonds of unsaturated fatty acids and inversely proportional to the pH of the subphase.
Schneider, Holman, and Burr (24) found that cis unsaturated fatty acids have expanded films. Molecular models of cis unsaturated fatty acids showed that double bonds present in the molecule cause it to bend which increased the area/molecule. Furthermore, Schneider et al. proposed that thermal agitation of fatty acids with increase unsaturation also expanded the area/molecule. However, trans monounsaturated films form more condensed films than the cis isomers. Adam (25) believed that the molecular structure of trans isomers allow them to come closer together than the cis isomers and form condensed films which Schneider et al. confirmed from molecular models of trans and cis isomers.

The force-area curves for oleic, linoleic, linolenic, and arachidonic acids were nearly identical. Furthermore these acids had the same collapse area, 32Å²/molecule and nearly the same interpolated area at 16 dynes/cm. Thus like long chain saturated fatty acids, long chain unsaturated fatty acids have the same force-area isotherms.

Other workers used surface film studies to determine the molecular structure of different fatty acids. Goddard and Morton (26) used surface chemistry techniques to show that a hemolytic acid isolated from horse brain formed expanded films similar to cis monounsaturated fatty acids. They proposed that the hemolytic acid was 11-cis-octadecenoic acid since the dihydroxyl derivatives of the hemolytic
acid and synthetic 11-cis-octadecenoic acid had the same force-area isotherms.

Anderson et al. (27, 28) isolated two long chain carboxylic acids from acid fast bacteria and proposed that the acids had a branched chain structure. Stallberg-Stenhagen and Stenhagen (29) found that the acids formed condensed monolayers typical of long chain saturated fatty acids while synthetic branched chain acids have expanded films. Thus, the molecular structure of different acids can be predicted from similarities seen in the force-area isotherms of unknown and synthetic acids.

Langmuir (15) showed that long chain saturated fatty acids have the same force-area isotherms while Schneider et al. (24) observed that naturally occurring unsaturated fatty acids also have similar force-area curves. Therefore different mixtures of fatty acid should have similar force-area isotherms as long as the percentage of saturated and unsaturated fatty acids is similar.

Cornwell, Heikkila, Bar, and Biagi (30) reported variations in the fatty acid composition and distribution of molecular species for erythrocyte phospholipids from different animals. However similar force-area isotherms were observed from erythrocyte phospholipids from these animals. Furthermore the apparent area/molecule for erythrocyte phospholipids calculated from the force-area isotherms of the total lipid was similar for all animals.
studied. Thus, there seems to be a control mechanism which maintains a constant area/molecule for the different phospholipid mixtures from mammalian erythrocytes.

The apparent area/molecule for phospholipids in erythrocytes is calculated from equation 1.

\[ A'_{PL} = \frac{A_{cell}}{m_{PL}} R - \frac{A_{chol}}{m_{chol}} \]  

(1)

where \( A'_{PL} \) is the apparent area of membrane phospholipids, \( A_{cell} \) and \( A_{chol} \) are the surface area of the cell and cholesterol respectively, \( R \) is the ratio of the lipid film area to cell area, and \( m_{PL} \) and \( m_{chol} \) are the molecules of phospholipid and cholesterol present in the film. This equation can be used to calculate the surface area of mammalian erythrocytes. The equation assumes that only cholesterol and phospholipids are present in the erythrocyte and the cholesterol/phospholipid ratio is similar for all mammalian species. The first assumption was correct since the force-area isotherms for the neutral lipid fraction and pure cholesterol were the same except at very low pressures. The second assumption was valid since the cholesterol/phospholipid ratio was nearly the same in all mammalian species studied. The similarity in the cholesterol/phospholipid ratio for mammalian erythrocytes is important since the condensing effect of cholesterol on phospholipid films varies with different molar ratios. The
Erythrocyte surface area can be calculated from the $m_{PL}$ and slope of equation 1. Since the cell surface area calculated in this manner agreed with the cell area values calculated from the cell diameter, it is apparent that force-area isotherms are the same for erythrocyte phospholipids of different animals (Table 2).

**TABLE 2**

RELATIONSHIP BETWEEN SURFACE AREA OF ERYTHROCYTE AND PHOSPHOLIPID CONTENT

<table>
<thead>
<tr>
<th>Species</th>
<th>$D(A)^a$</th>
<th>$A_{cell}^b$ $(\mu^2)$</th>
<th>$m_{PL} \times 10^7$</th>
<th>$A_{cell}^c$ $(\mu^2)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>8.5</td>
<td>152</td>
<td>24.4</td>
<td>145</td>
</tr>
<tr>
<td>Rat</td>
<td>7.5</td>
<td>118</td>
<td>15.8</td>
<td>93.7</td>
</tr>
<tr>
<td>Bovine</td>
<td>6.0</td>
<td>75.6</td>
<td>12.6</td>
<td>74.6</td>
</tr>
<tr>
<td>Sheep</td>
<td>5.2</td>
<td>56.8</td>
<td>10.6</td>
<td>62.9</td>
</tr>
</tbody>
</table>

$^a$ $D(A)$ is the cell diameter.

$^b$ $A_{cell}$ calculated at $2.1D^2$.

$^c$ $A_{cell}$ calculated as $59.3 m_{PL}$, where 59.3 is the slope of the regression line.

Quantitative Determination
Cholesterol and Cholesteryl Esters

Windaus (31) found that digitonin precipitated 3β-
hydroxysterols such as cholesterol and applied this reaction
to the macrogravimetric determination of cholesterol.
Grigaut (32) introduced a procedure for the quantitative
determination of cholesterol using the Liebermann-
Burchard reaction.

Autenrieth and Funk (33) proposed a method for
cholesterol determination which became a laboratory
standard for many years. This method differed from the
method of Grigaut (32) in that the color reagent was
added to a chloroform solution of the sterol and heated to
hasten color development.

Bloor (34) determined total cholesterol after extrac-
tion with alcohol-ether (3:1). However the values obtained
for cholesterol from the Bloor method were higher than
values obtained either from the Autenrieth and Funk method
or the macrogravimetric method. Kingsley and Schaffert (35)
proposed the use of a correction factor to obtain more
realistic values for total cholesterol while Kanter et al.
(36) suggested that cholesteryl acetate rather than chole-
sterol be used as the standard. Reinhold (37) reported
color development at 2°C was exclusively from cholesteryl
esters while at 38°C the color was from the total
cholesterol. In 1963 Webster (38) showed that cholesteryl esters gave more intense color than free cholesterol in the Liebermann-Burchard reaction. Therefore cholesterol determinations which do not include a hydrolytic step give high values for total cholesterol.

The methods of Schoenheimer and Sperry (39) and Abell et al. (40) use a saponification step before color development. The method of Schoenheimer and Sperry was developed in 1934 and used a digitonide precipitation before and after saponification with potassium hydroxide. The method became a laboratory standard and was used by investigators as a reference method to evaluate new methods. Since the method of Abell et al. does not require digitonide precipitation, the determination is quicker than the method of Schoenheimer and Sperry. The method of Abell et al., according to Tonks (41), has won general approval as the best reference method for the determination of total cholesterol. However cholesteryl esters cannot be determined directly from this method since saponification is required at the beginning of the method. The method of Sperry and Webb (42), a modification of the method of Schoenheimer and Sperry (39), is the preferred method for cholesteryl esters.

The mechanism for the Liebermann-Burchard reaction is unknown. The reaction, which is dependent on light, time,
and temperature, is inhibited by water. The concentration of cholesterol also effects the rate of color formation. Other sterols such as 7-dehydrocholesterol and lathosterol give approximately twice and four times as much color as cholesterol (43).

Since the maximum color development of the 625μ peak is time dependent, Clarke and Marney (44) and Schube (45) proposed that the yellow peak at 430μ be used. Since the yellow color is light sensitive, Kenny (46) proposed that the color be developed in the dark.

Huang et al. (47), Ness et al. (48), Ferro and Ham (49), and Kim and Goldberg (50) have reported direct methods for the Liebermann-Burchard reaction. However bilirubin causes an overestimation for the values of cholesterol (41). The interference caused by bilirubin is eliminated by saponification or digitonide precipitation. Therefore the direct methods are not as accurate as the method of Abell et al. (40) and Sperry and Webb (42). Pearson et al. (51) used para-toluenesulphonic acid to determine cholesterol. This method is simpler than the Liebermann-Burchard reaction since the reagents are more stable and the same color intensity is observed with free and esterified cholesterol. Watson (52) substituted 2,5-dimethyl-benzene-sulphonic acid and found that cholesterol and cholesteryl acetate gave the same color intensity.
Van Boetzelaer and Zondag (53) suggested that there is an initial formation of cholesteryl acetate when the sulphonlic acid methods for cholesterol determination are used.

Zlatkis, Zak, and Boyle (54) modified the Kiliani reaction to determine cholesterol. The original reagent, ferric chloride in sulfuric acid, was unstable but a dilute solution of ferric chloride in acetic acid is stable for at least one month. Martinek (55) added citric acid to the reagent and claimed the reagent was usable for two years. However the purity of acetic acid is important since glyoxylic acid contamination reacts with any proteins which are present and gives an intense, interfering color. MacIntyre and Ralston (56) recommended purification of the acetic acid by distillation from potassium dichromate. Zak (57) and Henly (58) recommended removal of protein either by organic solvents or strongly acid ferric chloride-acetic acid solution. The ferric ion-sulfuric acid method is more sensitive than either the Liebermann-Burchard or the sulfinic acid methods. Furthermore, the reaction is not time or light dependent. However, there are more possible sources of interference than with the other two methods.

Other sterols such as 7-dehydrocholesterol and lathosterol give approximately two and four times as much color as cholesterol in the Liebermann-Burchard reaction
(43). When cholesterol is exposed to air and light, it slowly oxidizes to form a mixture of products. Radin and Gramza (59) and Williams et al. (60) recommended that the dibromide method of Fieser (61) be used to purify cholesterol used as the primary standard since this procedure eliminates cholestanol, lathosterol, and some products of air oxidation.

**Quantitative Determinations of Triglycerides**

Triglycerides were calculated from the gravimetric difference between total lipids and the sum of phospholipids and free and esterified cholesterol (62). However, such substances as hydrocarbons, free fatty acids, and fat-soluble vitamins which are present in the total lipid fraction contributed to the values for triglycerides. Besides total lipids, total fatty acids and total esterified fatty acids have also been used.

The ester and glycerol determinations now permit direct quantitation of triglycerides. The ester determination was originally introduced as a qualitative test by Feigl et al. (63). Carboxylic esters react with alkaline hydroxylamine to form hydroxamic acids which form intense color complexes with ferric ions.

\[
\text{R} - \text{C} - \text{OR'} + \text{NH}_2\text{OH} \xrightarrow{\text{alkaline medium}} \text{R} - \text{C} - \text{NHOH} + \text{R'}\text{OH}
\]
Rapport and Alonzo (64) used a modified version of the method of Kornberg and Pricer (65) to determine acyl esters. Stern and Shapiro (66) described a modification which used ferric chloride while Snyder and Stephens (67) added the alkaline hydroxylamine reagent to dried triglycerides. Skidmore and Entermann (68) have reviewed the conditions used by other workers for the hydroxamic acid method. Antonis et al. (69) have developed an automated method for the colorimetric determination of acyl esters in serum.

Holla et al. (70) proposed a procedure to determine the ester content of triglycerides by quantitative gas-liquid chromatography. The ester groups are reduced to alcohols with lithium aluminum hydride and then quantitatively acetylated to form triacetin and fatty alcohol acetates. Besides the acyl ester determinations triglycerides are determined by enzymatic and chemical assays on the glycerol moiety. The colorimetric determination of glyceride-glycerol is based on its oxidation to formaldehyde with periodate according to the procedure of McFadyen (71) as modified by Lambert and Neish (72). The formaldehyde is quantitated colorimetrically after reaction with chromatropic acid. This reaction has been applied to manual
determinations of triglycerides by Van Handel and Zilversmit (73), Carlson and Wadström (74), and Christophe and Malthijs (75). Lofland (76) has automated the method. Hanahan and Olley (77) observed that high blank values are reduced by the addition of thiourea.

Kessler and Lederer (78) described a semiautomated fluorimetric method for the quantitation of triglycerides based on the Hantzsch condensation between an amine, diketone, and aldehyde. Fletcher (79) used the Nash modification of the Hantzsch reaction to determine triglycerides colorimetrically. Formaldehyde reacts with acetylacetone to form a yellow dihydrolutidine derivative which absorbs at 405 mp. Similarly the fluorimetric reaction of glycerol and O-aminophenol has been used to quantitate triglycerides (80).

Freeman et al. (81) have developed a semiautomatic analysis of serum triglycerides and cholesteryl esters by infrared absorption. Schlierf and Wood (82) used spot size measurements after thin layer chromatography (TLC) to quantitate triglycerides.

Kreutz (83) described a determination for triglycerides based on the enzymatic assay of glycerol with glycerokinase. Garland and Randle (84) and Printer et al. (85) have reported similar determinations. The assay is followed spectrophotometrically by recording the decrease in NADH absorbance at 340 mp. Laurell and Tibbling (86) have
reported on a fluorimetric determination of the same reaction. The enzymatic reactions are given below:

\[
glycerol + ATP \xrightarrow{\text{glycerokinase}} \alpha\text{-glycerol phosphate} + \text{ADP} \\
\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{pyruvate} + \text{ATP} \\
\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactic dehydrogenase}} \text{lactate} + \text{NAD}^+ 
\]

Antonis (87) has automated the enzymatic fluorimetric assay of glycerol.

Frings and Pardue (88) have developed a method which uses glycerol dehydrogenase coupled to a diaphorase oxidation-reduction system. However other polyhydroxyl compounds such as propylene glycol interfere with the reaction. Williams and Soeling (89) mention that potassium hydroxide may contain glycerol contamination which interferes with the enzymatic and colorimetric determinations on the glycerol moiety.

Quantitative Determinations of Phospholipids

Total phospholipids are quantitated from the determination of phosphorus content of lipid extracts if nonlipid phosphorus is removed. Most phospholipid colorimetric
determinations are modifications of the method of Fiske and Subbarow (90). In this method phosphate is converted to phosphomolybdic acid and then reduced to a molybdenum blue complex. King (91) modified the method with the introduction of the perchlorate ashing step which converts organic phosphates to inorganic phosphates.

Berenblum and Chain (92) extracted phosphomolybdic acid with isobutanol to eliminate the interference of tissue contaminants in the colorimetric reaction. Martin and Doty (93) used an equal mixture of isobutanol and benzene to extract the phosphomolybdic acid which decreased the time of the extraction step.

Boltz and Mellon (94) quantitated phospholipids as phosphomolybdic acid without further reduction. Soyenkoff (95) noticed that phosphomolybdate caused a color change in the dye, quinaldine red, and used this change to determine phospholipids.

Lowry et al. (96) used ascorbic acid in the reduction step and noticed a six fold increase in the sensitivity of the method of Fiske and Subbarow (90). Bartlett (97) ashed the phospholipids with sulfuric acid and hydrogen peroxide which he said decreased the sensitivity to variations in reagents and improved the color stability.

While the method of Bartlett (97) was designed for column chromatograph, Rouser et al. (98) introduced a method for TLC. Rao et al. (99) used basic alumina to
chromatograph phospholipids before they determined phosphorus by the method of Rouser et al. Since basic alumina forms water soluble salts during digestion, centrifugation of the absorbent is eliminated.

Neskovic (100) and Christensen Lou and Clausen (101) described methods for the photodensitometry determination of phospholipids from TLC. Shibuya et al. (102) have used alcoholic sulfuric-periodic acid to quantitate lecithin. Rosenthal and Han (103) adapted the method to phosphoglycerides containing two acid hydrolyzable groups. Cardiolipin reacts only partially, while sphingomyelin, and diether lecithin, fail completely to react.

Thaxton and Bowie (104) hydrolyzed lecithins to choline with phospholipase D. The choline was precipitated as the reineckate salt and determined colorimetrically by the method of Kates (105).

Rhee and Dugan (106) studied the effects of acid concentration in determinations of lipid phosphorus by the ascorbic acid reducing method of Rouser et al. (98) and the aminonaphthosulfonic acid reducing method of Parker (107). They observed that phosphorus color development in both methods is acid dependent. The acids used were concentrated sulfuric acid, perchloric acid, and a 1:1 mixture of the two acids since these are the most commonly used acids for digestion of phospholipids. A graph of the acid concentration vs. the absorbance showed a plateau where
the acid concentration did not affect the absorbance. The pH of the acid in the plateau region was 3.8 - 4.0. Rhee and Dugan believe that perchloric acid and mixtures of perchloric and sulfuric acids should be used in the digestion step since the ashing is rapid and the range of acid for the phosphorus determination is wide.

The μmoles of cholesterol, phospholipids, and triglycerides required to give an absorbance of 0.400 optical density (OD) units is given in Table 3.

Isotope Dilution of Lipids

When a radioactive compound is mixed with a similar unlabelled sample, the mixture has a lower specific activity than the labelled compound. If the specific activity of the labelled compound and sample can be determined, then the quantitation of the sample is possible. The specific activity of the standard is defined as

$$SA_{STD} = \frac{cpm}{W_{STD}}$$  \hspace{1cm} (2)

where $SA_{STD}$ is the specific activity of the standard, cpm is the counts per minute and $W_{STD}$ is the weight in gm of standard used although μmoles, absorbance, or similar measurements are sometimes used. When the labelled standard is diluted with the unlabelled sample, the specific activity of the mixture is:
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Method</th>
<th>pmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Abell et al. (40)</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Kenny (46)</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>Kim and Goldberg (50)</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>Pearson et al. (51)</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>Watson (52)</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>Zlatkis et al. (54)</td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td>Trinder (108)</td>
<td>0.556</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Fiske and Subbarow (90)</td>
<td>0.906</td>
</tr>
<tr>
<td></td>
<td>Berenblum and Chain (92)</td>
<td>0.226</td>
</tr>
<tr>
<td></td>
<td>Lowry et al. (96)</td>
<td>0.151</td>
</tr>
<tr>
<td></td>
<td>Bartlett (97)</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>Rouser et al. (98)</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td>Rosenthal and Han (103)</td>
<td>0.250</td>
</tr>
<tr>
<td></td>
<td>Parker (107)</td>
<td>0.136</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Stein and Shapiro (66)</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>Synder and Stephens (67)</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>Skidmore and Entermann (68)</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>Antonis et al. (69)</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>VanHandel and Zilversmit (73)</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>Carlson and Wadström (74)</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>Fletcher (79)</td>
<td>0.450</td>
</tr>
<tr>
<td></td>
<td>Mendelsohn and Antonis (80)</td>
<td>0.051b</td>
</tr>
<tr>
<td></td>
<td>Garland and Randle (84)a</td>
<td>0.070b</td>
</tr>
<tr>
<td></td>
<td>Pinter et al. (85)c</td>
<td>0.135</td>
</tr>
</tbody>
</table>

aThe determination is not linear above an absorbance of 0.280 OD units.
bThe pmoles required for OD of 0.200.
cThe determination is not linear above 0.500 OD units.
Numbers in parenthesis are reference numbers.
where $SA_{SAM}$ is the specific activity of the mixture of labelled standard and unlabelled sample and $W_{SAM}$ is the weight of the sample.

The isotope dilution formula is derivated from equation 2 and 3:

$$SA_{SAM} = \frac{cpm}{W_{STD} + W_{SAM}}$$  \hspace{1cm} (3)

$$SA_{SAM} = \frac{cpm}{W_{STD} + W_{SAM}}$$  \hspace{1cm} (4)

$$(SA_{SAM}) (W_{STD} + W_{SAM}) = cpm = (SA_{STD}) (W_{STD})$$  \hspace{1cm} (5)

$$W_{SAM} = W_{STD} \left( \frac{SA_{STD} - SA_{SAM}}{SA_{SAM}} \right)$$  \hspace{1cm} (6)

$$W_{SAM} = W_{STD} \left( \frac{SA_{STD}}{SA_{SAM}} - 1 \right)$$  \hspace{1cm} (7)

When the specific activity of the standard is ten times greater than that of the sample, then the accuracy of the technique is maximized.

Factors which affect the specific activity of the standard or sample decrease the accuracy of the method. The standard must be chemically pure, radiochemically stable during storage, and chemically identical with the diluting material during the experiment. Any changes in the specific activity of the standard affect the value obtained for the weight of the sample from the isotope dilution equation. Thus the specific activity of the
standard must be routinely redetermined to insure that it has not changed. Rosenblum (109) showed that vitamin B\textsubscript{12}-\textsuperscript{60}Co stored in aqueous solutions at room temperature had a ten percent loss in specific activity per year while only a 1.8 percent yearly loss was noticed when it was kept at 4°C.

The complete mixing of labelled and unlabelled samples is required at the beginning of the experiment. If a disproportionate amount of labelled compound is lost, the specific activity of the mixture decreases. Thus, a high value for the amount of sample present is obtained from the isotope dilution formula. If a disproportionate amount of sample is lost, then the specific activity of the mixture increases and a low value for the amount of sample is obtained.

The purity of the final product is also important since any extraneous material which affects the specific activity of the sample causes inaccurate results. However, the quantitative recovery of the mixture is not required since the chance of losing either labelled or unlabelled material is identical if the sample is completely mixed. The isotope dilution method is highly suitable for quantitation of small samples because it is easier to assure the purity of small samples than the quantitative recovery.

In 1934 the isotope dilution technique was introduced by Hevesy and Hofer (110) who used deuterium oxide to
determine the amount of water in the human body. The isotope dilution method was not fully accepted until Rittenberg and Foster (111) used $^{15}$N to determine the amino acid content of various proteins. They also used deuterium labelled palmitic acid to quantitate the amount of this acid in animal fat. Later Chargaff, Ziff, and Rittenberg (112) used $^{15}$N to study the composition of tissue phospholipids. Similar early studies were done on other biochemical compounds and these studies were reviewed by Pinajian, Christian, and Wright (113).

Hitchcock, Morris, and James (114) used isotope dilution techniques to show that D-2-hydroxylpalmitic acid is formed in the α-oxidation. They mixed an excess of synthetic methyl D-2-hydroxylpalmitate with the methyl ester of 2-hydroxylpalmitic acid -1$^{14}$C synthesized in vitro. After recrystallization the specific activities of the crystals and the mother liquor were the same. When the process was repeated with synthetic methyl L-2-hydroxylpalmitic acid present in excess, most of the radioactivity remained in the mother liquor. Since the radioactivity was found only in the crystals of the D enantiomer, the authors concluded that D-2-hydroxylpalmitic acid was synthesized in vitro.

Seyama, Yamakewa, and Komai (115) used an isotope dilution analysis to determine individual sphingoglycolipids. They selected this method since the content of
sphingoglycolipids in the total lipid sample was small and the quantitative isolation of these lipids involved a tedious procedure. The isotope dilution method overcame these difficulties since the method required the purification rather than the quantitative recovery of the sample. They noticed that sphingoglycolipids became labile when labelled with tritium both immediately after synthesis and during storage. The decomposition of the tritiated sphingoglycolipids decreased the specific activity of the standard which caused the quantitation by isotope dilution to be invalid. When the tritiated sphingoglycolipids were diluted with unlabelled sphingoglycolipids immediately after tritiation, there was no observed loss in the specific activity.

Kritchevsky, Tepper, and Shapiro (116) quantitated serum cholesterol by isotope dilution and four different colorimetric methods. They found the best comparison between the isotope dilution method and colorimetric methods which involved digitonide precipitation.

Newshole and Taylor (117) proposed a method to quantitate glycerol and other metabolites by using both isotope dilution and enzymatic techniques. The method required a compound which is isotopically labelled, an enzyme that reacts specifically with the compound, and the isolation of the product of the enzymatic reaction.
CHAPTER II
STATEMENT OF THE PROBLEM

A simple microdetermination of different lipids is desirable since some tissues are either very small or have low concentrations of one or more lipids. For example, it is extremely difficult to examine the intima from a human coronary artery which weighs 15-20 mgs. Similarly the lipid composition of individual aortic plaques cannot be investigated without a micromethod.

Lipids occupy large surface areas when they are spread as monolayers on an aqueous subphase. A typical lipid such as cholesterol has a surface area of 40Å²/molecule or 2409 cm²/µmole. Surface areas as small as 20 to 50 cm² are easily measured with a film balance. Thus lipid micro-analysis is readily accomplished if surface area measurements are adapted as an analytical procedure. The surface area of a pure compound is directly proportionate to the amount of that compound added to the trough

\[ A = K \mu M \]  

(8)

where \( A \) is the area in square centimeters (cm²), \( K \) is the proportionality constant (cm²/µmole), and \( \mu M \) is the amount of lipid (µmoles).
Temperature, surface pressure, nature of the subphase, molecular structure, and fatty acid composition also affect the surface area. All of these except molecular structure and fatty acid composition are readily controlled. However, these two factors do not interfere with surface area quantitation of pure compounds. Changes in the composition of fatty acids occur with tissue triglycerides which are mixtures of molecules with different fatty acid composition while tissue phospholipids vary in both molecular structure and composition. If structure and fatty acid composition vary significantly, then surface area quantitation is impossible. The sensitivity of the surface area to change is determined by obtaining the area/μmole. If the respective area/μmole for phospholipids and triglycerides from all tissues is nearly the same, then variations in structure and fatty acid composition in these complex lipids do not affect the surface area. If the proportionality factor for a lipid varies for different tissues but is constant for each tissue, then individual tissue constants are required for surface area quantitation of each lipid. Therefore the purpose of this study is to obtain the proportionality constants for phospholipids, triglycerides, and cholesterol of different tissues and thus to determine the practicality of surface area measurements for micro-quantitation of lipids.

Although 0.008 μmoles of cholesterol are quantitated
from surface area measurements, it is often difficult to isolate and purify quantitatively samples of such small amounts. If isotope dilution is used with surface area measurements, the need for quantitative recovery of lipids from multistep extraction and isolation procedures are eliminated. Thus isotope dilution which requires only purity of the sample makes lipid quantitation by surface measurements of small tissue samples practical.
CHAPTER III
EXPERIMENTAL METHODS

Material

Demineralized double distilled water which had less than 0.1 ppm impurities expressed in terms of NaCl was prepared by the Ohio State University Reagent Laboratory. The water was distilled, passed through a demineralizer, and then redistilled. Castor oil was purchased either from E. R. Squibb Co. (New York, New York) or Eastman Organic Chemicals (Rochester, New York) which also supplied tricresyl phosphate. Hexane, used as the spreading solvent, was prepared by the method described by Heikkila, Kwong, and Cornwell (18).

Cholesterol-4-\(^{14}\)C (50 pcuries/\(\mu\)mole) and tri-palmitin-carboxyl-\(^{14}\)C (50 pcuries/\(\mu\)mole) were purchased from New England Nuclear (Boston, Mass.) while cholesterol-4-\(^{14}\)C palmitate (21.2 pcuries/\(\mu\)mole) was purchased from the Radiochemical Centre (Amersham, England). 2,5-diphenyloxazole (PPO) and 1,4-bis \(\left[2-(5\text{-phenyl-oxazolyl)}\right]\) benzene (POPOP) were purchased from either Packard Instrument Co. (Downers Grove, Ill.)
or Tracerlab (Waltham, Mass.).

Cholesterol and methyl oleate used as standards for the colorimetric determinations were purchased from either the Hormel Institute (Austin, Minn.) or Applied Science Laboratories (State College, Penna.) while dibasic sodium phosphate which was the standard for the phosphorus determination was purchased from Baker Chemical Co. (Phillipsburg, New Jersey). Acetic anhydride used in the cholesterol determination was purchased from Fisher Scientific Co. (Fairlawn, N.J.) and glacial acetic acid from E. I. DuPont De Nemours and Co. (Wilmington, Del.). The perchloric acid and ferric perchlorate (non-yellow) used in the ester determination were purchased from G. Frederick Smith Co. (Columbus, Ohio). Ascorbic acid was purchased from Merck and Co. (Rahway, N.J.). All other chemicals used in the colorimetric determinations were purchased from J. T. Baker Co. (Phillipsburg, N.J.).

Analytical grade diethyl ether was purchased from Mallinckrodt Chemical Works (St. Louis, Mo.), while all other solvents were purchased from J. T. Baker (Phillipsburgh, N. J.). Disodium dihydrogen ethylenediaminetetra-acetate dehydrate (EDTA) was purchased from G. Frederick Smith Co. (Columbus, Ohio). Activated silicic acid (Unisil) was purchased from Clarkson Chemical Co. (Williamsport, Penna.) and Silica Gel H was purchased from Brinkmann Instrument, Inc. (Westburg, N. Y.).
Extraction and Purification of the Lipids

Bovine blood was obtained from a local slaughter house while human blood was drawn from three healthy males. EDTA was added to all blood samples to prevent coagulation. The blood was centrifuged at 4°C to separate the plasma from the erythrocytes. The plasma samples were added slowly to 10 volumes of methanol which was continuously stirred. After 20 minutes the samples were filtered through Whatman No. 1 filter paper, the residue reextracted with 10 volumes of chloroform-methanol (1:1) for 20 minutes, filtered, and reextracted again with 10 volumes of chloroform-methanol (2:1) for 20 minutes. The red blood cells were washed three times with five volumes of cold isotonic saline and extracted with chloroform-isopropanol (7:11) (118).

Normal liver, adrenal, and adipose tissues were obtained at the autopsies of subjects whose death was accidental and stored at -20°C until used. The tissues were extracted successively with ten volumes of chloroform-methanol (2:1), chloroform-methanol (1:1), and methanol except for the adipose tissue for which the methanol extract was omitted. Extracts were combined, the solvent was removed under reduced pressure, and excess water was removed and the benzene azeotrope. The crude lipid in all
extractions was dissolved in chloroform-methanol (2:1), and washed according to the method of Folch et al. (119). The crude lipids were placed on a 10 g Unisil column (2.0 cm i.d.). The neutral lipids were eluted with 200 ml chloroform, glycolipids and colored material with 100 ml acetone, and phospholipids with 200 ml methanol. The neutral lipids were separated on 0.5 mm Silica Gel H plates with hexane-diethyl ether (90:10) as the solvent system. When the solvent reached within one inch of the top of the plate, the plate was removed, air dried, and placed in a second solvent system, diethyl ether-hexane (70:30), and was run to the bottom of the triglyceride standard. The second solvent system increased the Rf of the cholesterol band. The plate was sprayed with ethanolic 2',7'-dichlorofluorescein and visualized in ultraviolet light. The bands corresponding to the standard were scraped off the plate, placed on 2-3 g Unisil columns, and eluted with ether. After evaporation of the ether, the purified lipid fractions were diluted to a known volume in hexane.

An alternative method was used for larger neutral lipid samples. Cholesteryl esters and triglycerides were eluted from the Unisil column with 10 percent diethyl ether in hexane, and cholesterol with 25 percent diethyl ether in hexane. The cholesteryl esters and triglycerides were separated on TLC. All fractions eluted from the column
were monitored for purity routinely by TLC.

Lipid phosphorus was determined by the method of Lowry et al. (96). Triglycerides were determined by the method of Snyder and Stephens (67). The method of Abell et al. (40) was used for cholesterol determinations in a slightly modified form. The saponification step was extended to 90 minutes at 75°C since the reaction was incomplete at the lower temperatures (37°C) and shorter time (55 minutes) which they used.

**Surface Area Measurements**

The Langmuir trough used in this study was the same as described by Heikkila, Deamer, and Cornwell (19). The trough was filled with 0.02 M NaCl in all experiments and a plexiglass floating barrier was placed on the surface.

An aliquot of a hexane solution of lipid made to a known volume was pipetted on one side of the barrier. After a few seconds to allow the hexane to evaporate, a piston oil was applied to the other side. The barrier moved until lens of excess piston oil appeared. Then the surface area of the film was measured with a stainless steel ruler attached to the side of the trough. The time of the experiment was less than one minute. After the experiment the surface on each side of the barrier was cleaned by suction until the barrier rested against the side of the
trough.

The surface pressure of both the sample and the excess piston oil monolayers were identical and this pressure is the equilibrium spreading pressure of the piston oil. The equilibrium spreading pressure is defined as the pressure where the monolayer is in equilibrium with the stable bulk phase (liquid or solid) under the conditions of the experiment. In these experiments castor oil was used as the piston oil for the surface determinations of cholesterol and phospholipids, while tricresyl phosphate was used for triglycerides. The surface pressure exerted by the piston oils was measured several times during the course of the experiments and agreed with the literature values (120).

The change from a stronger to weaker piston oil for triglycerides was necessary since the interactions of the polar end of the triglycerides molecules with the water molecule was not as great as with phospholipid and cholesterol. Therefore triglycerides do not form as stable monolayers as phospholipid and triglycerides. If castor oil was applied as the piston oil to a triglyceride monolayer, the monolayer would have collapsed. There are several other piston oils besides those used in the study (Table 4).
### TABLE 4
**PISTON OILS**

<table>
<thead>
<tr>
<th>Material</th>
<th>Approximate pressure on water at 25°C (dynes/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic Acid</td>
<td>30</td>
</tr>
<tr>
<td>Ethyl myristate</td>
<td>20</td>
</tr>
<tr>
<td>Castor Oil</td>
<td>17</td>
</tr>
<tr>
<td>Triolein</td>
<td>15</td>
</tr>
<tr>
<td>tri-m-tolyl phosphate (tricresyl phosphate)</td>
<td>9</td>
</tr>
<tr>
<td>Mixtures of ethyl laurate and heavy mineral oil</td>
<td></td>
</tr>
<tr>
<td>60% ethyl laurate</td>
<td>17</td>
</tr>
<tr>
<td>40% ethyl laurate</td>
<td>14</td>
</tr>
<tr>
<td>30% ethyl laurate</td>
<td>11</td>
</tr>
<tr>
<td>15% ethyl laurate</td>
<td>7</td>
</tr>
</tbody>
</table>


**Isotope Dilution Method**

Radioactive and unlabelled carrier compounds were spotted on 0.25 mm Silica Gel H plates and developed in hexane-diethyl ether (90:10) to determine the purity of the radioactive compounds. The radiochromatograms obtained
from the radioactive plate scanner showed each compound was radiochemically pure.

Aliquots of each radioactive compound were taken to prepare the labelled standard used in the isotope dilution experiments. The triglyceride standard was prepared by adding approximately equal umolar aliquots of four purified adipose triglyceride samples to an aliquot of tripalmitin-carboxyl-\(^{14}\text{C}\). The labelled standard was made to volume in hexane since 1 ml aliquots of pure hexane did not quench the external standard. One ml aliquots of each radioactive solution were counted in 15 ml of scintillation mixture in a Packard Tri Carb liquid scintillation spectrometer for 10,000 counts. The surface area was obtained with other aliquots. A trap was attached to the water suction hose to prevent radioactive contamination of local sewage when the surface of the subphase was cleaned. The area, \(\text{cm}^2_{\text{STD}}\), and the cpm determined the specific activity of the labelled standard. Similar procedures were repeated to prepare the labelled cholesterol and cholesteryl palmitate standards. The specific activity used in these experiments is defined as:

\[
\text{SA}_{\text{STD}} = \frac{\text{cpm}}{\text{cm}^2_{\text{STD}}} \quad (9)
\]

Therefore the isotope dilution formula becomes:

\[
\text{cm}^2_{\text{SAM}} = \text{cm}^2_{\text{STD}} \left( \frac{\text{SA}_{\text{STD}}}{\text{SA}_{\text{SAM}}} - 1 \right) \quad (10)
\]
where \( \text{cm}^2_{\text{SAM}} \) and \( \text{cm}^2_{\text{STD}} \) are the square centimeters of sample and standard respectively obtained from surface area measurements.

In a typical isotope dilution experiment, the labelled lipid was added to give approximately 500 cpm/ml in the final solution and make an insufficient contribution to the total area of the mixture. In the experiments used to check the validity of the method, unlabelled lipid samples were purified before mixing with the radioactive standards. Different aliquots were taken for counting and area determinations for the specific activity measurements. Cholesteryl esters were saponified to cholesterol before counting and area measurement. When crude lipids were used the lipids were purified as mentioned earlier before the specific activity was determined. The \( \text{cm}^2_{\text{SAM}} \) was calculated from the isotope dilution formula and was divided by the proportionality constant to obtain the \( \mu \)moles in the sample.

The scintillation mixture consisted of 4 g of PPO and 0.1 g of POPOP in 1,000 ml toluene.
CHAPTER IV
RESULT AND DISCUSSION

Cholesterol and Cholesteryl Esters

The tissue sources for free and esterified cholesterol were bovine plasma, human plasma, erythrocytes, and adrenal glands. The entire human tissue and blood samples were used to determine the proportionality constant while each bovine plasma sample was divided into three aliquots before they were individually extracted and independent area/µmole determinations attempted. The cholesteryl ester samples were saponified since Kwong, Heikkila, and Cornwell (121) reported that saturated and unsaturated cholesteryl esters are not sufficiently polar to form stable monolayers. When saturated cholesteryl esters were spread and compressed, rigid and sometimes cloudy films with characteristics of scum-like multilayers appeared. However, unsaturated cholesteryl esters formed expanded curves after equilibrating at the air-water interface for several hours. Kwong et al. believed the expanded curve was caused by the autoxidation of the unsaturated fatty acyl group.

The Abell et al. (39) saponification procedure was
modified by increasing the temperature to 75°C for 1.5 hours because the saponification was incomplete when monitored by TLC at the shorter time (55 minutes) and lower temperature (37°C) which they reported. Transesterification with sodium methoxide is an alternative method to form cholesterol from cholesteryl esters (122).

The area/µmole for free cholesterol and esterified cholesterol saponified to cholesterol were 2472 cm²/µmole and 2437 cm²/µmole respectively (Table 5 and 6).

Since cholesterol is a single component film, the discrepancies in the area/µmole from different sources indicate the presence of a surface area occupying contaminant or errors in experimental techniques. Since the area/µmole is determined from surface area and colorimetric measurements, any contaminants which change one of these parameters vary the area/µmole. Therefore impure samples containing other surface active lipids have larger areas/µmole. Thus, the purity of each sample must be checked routinely. Similarly any extraneous contaminants such as hair oil, vacuum grease, and lubrication oil which increase the surface area cause higher areas/µmole. Although such contaminations are possible with other lipid samples, their presence is more noticeable with cholesterol since it has an area/µmole that is the most invariant.

After the surface area is obtained, the number of µmoles of cholesterol is determined from either the
### TABLE 5
PROPORTIONALITY CONSTANTS FOR CHOLESTEROL

<table>
<thead>
<tr>
<th>Source</th>
<th>cm²/µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Plasma</td>
<td>$2595 \pm 42(6)^a$</td>
</tr>
<tr>
<td></td>
<td>$2445 \pm 135(3)$</td>
</tr>
<tr>
<td></td>
<td>$2443 \pm 64(3)$</td>
</tr>
<tr>
<td>Human Erythrocyte</td>
<td>2480 (1)</td>
</tr>
<tr>
<td>Human Plasma</td>
<td>2443 (1)</td>
</tr>
</tbody>
</table>

*Mean ± standard derivation (SD). Numbers in parenthesis indicate number of determinations.*

### TABLE 6
PROPORTIONALITY CONSTANTS FOR CHOLESTEROL FROM CHOLESTERYL ESTERS

<table>
<thead>
<tr>
<th>Source</th>
<th>cm²/µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Plasma</td>
<td>$2378 \pm 145(6)^a$</td>
</tr>
<tr>
<td></td>
<td>$2326 \pm 97 (3)$</td>
</tr>
<tr>
<td></td>
<td>2348 (1)</td>
</tr>
<tr>
<td>Human Plasma</td>
<td>2463 (1)</td>
</tr>
<tr>
<td>Human Adrenal Gland</td>
<td>2502 (1)</td>
</tr>
</tbody>
</table>

*Mean ± SD. Numbers in parentheses indicate number of determinations.*
standard curve (page 75) or by multiplying the surface area by the proportionality constant, \( \text{cm}^2/\mu\text{mole} \), which is the slope of the standard curve. The \( \mu \)moles of phospholipid and triglyceride are determined in a similar manner.

**Triglycerides**

The other neutral lipid fraction studied was the triglycerides from human plasma, liver, adrenal, and adipose tissues which were obtained at the autopsies of subjects whose death was accidental. The neutral lipid fractions from plasma, liver, and adrenal tissues were divided in half before the triglycerides were separated on TLC and had independent determinations of the area/\( \mu \)mole. Two adipose tissues had the proportionality constant determined on three aliquots of triglycerides separated from the total lipid while only two aliquots were used for the other two adipose tissues. The proportionality constant for the triglycerides from different tissues is given in Table 7. Since the area/\( \mu \)mole for triglyceride from all tissues sources showed remarkable similarity, the use of a single proportionality constant is feasible for surface area quantitation.

Since triglycerides are triesters of glycerol, the only variation in different triglycerides is in the fatty acid composition. Therefore any change in the proportion-
TABLE 7

PROPORTIONALITY CONSTANTS FOR TRIGLYCERIDES

<table>
<thead>
<tr>
<th>Source</th>
<th>cm²/µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose</td>
<td>6568 ± 53 (3)a</td>
</tr>
<tr>
<td></td>
<td>6613 (2)</td>
</tr>
<tr>
<td></td>
<td>6326 ± 72 (3)</td>
</tr>
<tr>
<td></td>
<td>6386 (2)</td>
</tr>
<tr>
<td>Liver</td>
<td>6106 ± 247 (4)</td>
</tr>
<tr>
<td></td>
<td>6556 ± 206 (4)</td>
</tr>
<tr>
<td>Adrenal</td>
<td>6480 ± 76 (4)</td>
</tr>
<tr>
<td>Plasma</td>
<td>6359 (2)</td>
</tr>
</tbody>
</table>

*aMean ± SD.
Numbers in parenthesis indicate number of determinations.

Proportionality constant for triglycerides is the difference in the percentage of saturated and unsaturated fatty acids. For example, tristearin and tripalmitin have molecular areas of 60Å²/molecule at surface pressures above a few dynes/cm (123). Since the condensed films of stearic acid have a molecular area of 20Å²/molecule, the molecular area of 60Å²/molecule for tristearin and tripalmitin indicates that only the acyl chain contributes to the area. Similarly, the area of triolein is approximately the area occupied by three oleoyl chains. Merker and Daubert (124) observed
that triglycerides containing short chain fatty acids have expanded films at room temperature.

Although the percentage of saturated and unsaturated fatty acids for triglycerides from different tissues varied, the difference was not sufficient to affect the proportionality constant. For example, liver and adrenal contained 43 percent and 26 percent saturated fatty acids respectively but the proportionality constant for triglycerides from the two tissues is the same. Table 8 gives the percentage of saturated and unsaturated fatty acids for triglycerides from different tissues of these subjects and percentages obtained by other workers for the same tissues. Thus small fluctuations in the percentage of saturated and unsaturated fatty acids do not disturb the proportionality constant.

Kaunitz, Johnson, and Belton (125) analyzed human adipose and liver tissue to determine the effect disease and nutritional states at death have on the fatty acid composition of triglycerides. The percentage of saturated and unsaturated fatty acids of liver and adipose triglycerides were nearly identical in all disease and nutritional states. Furthermore the percentages were similar to those obtained from liver and adipose tissue of accidental death subjects. Laudat et al. (126) noticed a similar saturated to unsaturated ratio in the plasma triglycerides of normal and essential familial
TABLE 8
PERCENTAGE OF SATURATED AND UNSATURATED FATTY ACIDS OF NORMAL HUMAN TISSUE

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% Saturated</th>
<th>% Unsaturated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.0</td>
<td>57.0</td>
</tr>
<tr>
<td>Adrenal&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.3</td>
<td>73.7</td>
</tr>
<tr>
<td>Adrenal (127)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.8</td>
<td>67.2</td>
</tr>
<tr>
<td>Plasma&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.7</td>
<td>61.3</td>
</tr>
<tr>
<td>Plasma (126)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.9</td>
<td>63.8</td>
</tr>
<tr>
<td>Plasma (149)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.3</td>
<td>66.6</td>
</tr>
<tr>
<td>Adipose&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.2</td>
<td>69.8</td>
</tr>
<tr>
<td>Adipose (129)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.4</td>
<td>59.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Panganamala, R. V., unpublished observations.
<sup>b</sup>Number in parenthesis is reference number.

Hypercholesterolemia subjects. Takayasu et al. (127) reported the same similarity in the triglycerides from normal and adenoma adrenals while Antonini et al. (128) and Kingsburg et al. (129) observed that the percentage of saturated fatty acids did not vary in adipose tissue of normal and atheroma subjects. Thus the quantitation of adipose, liver, plasma, and adrenal triglycerides from normal and diseased subjects is possible with the same proportionality constant.
Several workers have altered the triglyceride fatty acid composition of different organs by controlling the diet. Takayasu et al. (127) reported that oleic acid represented 55 percent of the rat adrenal triglycerides in fat deficient diets. In the control diet oleic acid represented only 38 percent of the fatty acids while linoleic acid, which was absent in the adrenal triglycerides of the rats on the fat free diet, represented 19 percent of the fatty acids in the control rats. However, the ratio of saturated to unsaturated fatty acids was 1 to 3 for rats kept on either diet.

Catala and Brenner (130) reported on the fatty acid composition of triglycerides from different organs of rats kept on either fat free diets or fat free diets supplemented with methyl linoleate. Their data showed that the percentage of saturated fatty acids of triglycerides from epirenal fat, liver, and a combined pool of heart, kidney, lung, and pancreas was not affected by the diet.

Kaunitz (131) reported that triglycerides of adipose, liver, heart, and kidney from rats fed butter or lard, diets low in linoleic acid, had approximately 33 percent saturated acids. The triglycerides of the same organs from rats fed corn oil or soybean oil, high in linoleic acid, had 20 percent saturated fatty acids. Kaunitz mentioned that brain triglycerides had 30 percent saturated fatty acids on all diets. The rats used by Kaunitz
were kept on the different diets for twenty months while those used by Catala and Brenner (130) were fed methyl linoleate for one month after being on the fat free diet for 70 days. Privett et al. (132) reported that rat adipose tissue exhibited a six month lag before the triglycerides reflected dietary alterations. Thus the data of Kaunitz reflected the prolonged effect of a high linoleate diet.

The effects of feeding unsaturated fatty acids to men for five years was studied by Dayton, Hashimoto, Dixon, and Pearce (133). A 6 percent decrease in the level of adipose saturated fatty acids was noticed in the men fed the linoleate diet when compared to men on the normal diet. The blood triglyceride of men on the linoleate diet showed a decrease of 10 percent in the level of saturated fatty acids over three years.

Antonini et al. (128) reported human adipose saturated fatty acid levels 6 percent lower than other investigators. Since the study occurred in Italy, Antonini et al. attributed the lower saturated fatty acid levels to the high content of olive oil found in the Italian diet. Kaunitz (131) also reported lower levels of saturated fatty acids in the adipose triglycerides of rats kept on olive oil for 20 months.

Since the area/μmole of all unsaturated fatty acids is similar, changes in the unsaturated diets for different
experiments should not affect the proportionality constant. Although the percentage of saturated fatty acids is lower in high unsaturated diets, the change in the percentage from normal diets may not be sufficient to cause a variation in the proportionality constant. Furthermore Hirsch et al. (134) pointed out that the turn over time for human adipose fatty acids is between one to two years. Thus small daily changes in the diet do not affect the proportionality constant for triglycerides.

**Phospholipids**

The proportionality constants of phospholipids from normal human plasma, erythrocytes, adrenal gland, liver, and bovine plasma were determined. The entire human plasma and red blood cell samples were used to determine the proportionality constant while each bovine plasma sample was divided into three aliquots which were individually extracted and had independent determinations of the area/µmole. Phospholipids were separated from the total lipid extract of two adrenal tissues before the proportionality constant was determined. The total lipid of the third tissue was divided in half before isolation and determination of the area/µmole of the phospholipids. The proportionality constant for two liver samples was determined on two and four aliquots of phospholipids separated from
the total lipid before independent determinations of the area/µmole was attempted. The results of the determination of the proportionality constants for the phospholipids are given in Tables 9 and 10.

The average cm²/µmole for blood and tissue phospholipids, liver and adrenal, was 5150 cm²/µmole and 4832 cm²/µmole respectively. The surface area quantitation of these phospholipids required two distinct proportionality constants. Since the grand mean of the proportionality constants differs from the individual proportionality constants by 3 percent, surface area quantitation of phospholipids using the grand mean proportionality constant is possible if less accurate determinations are tolerable.

The difference in the proportionality constant between tissue and blood phospholipids emphasizes the need to obtain the area/µmole for each new tissue examined and repeated redeterminations of the proportionality constants for all phospholipids.

The reason for the difference in the proportionality constant for blood and tissue phospholipids is unknown but genuine. The t test showed a difference in the area/µmole between tissue and blood phospholipids at 0.01 level of significance (Table 11 and Table 12).

Since glycolipids are surface active molecules, they interfere in the determination of the proportionality constant for phospholipids. Therefore extreme care must
### TABLE 9

**PROPORTIONALITY CONSTANT FOR BLOOD PHOSPHOLIPIDS**

<table>
<thead>
<tr>
<th>Source</th>
<th>cm²/µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bovine Plasma</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5121±76 (3)</td>
</tr>
<tr>
<td></td>
<td>5258±104 (9)</td>
</tr>
<tr>
<td></td>
<td>4974±56 (3)</td>
</tr>
<tr>
<td></td>
<td><strong>5117±142 (3)</strong></td>
</tr>
<tr>
<td><strong>Human Plasma</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5070 (2)</td>
</tr>
<tr>
<td></td>
<td>5260 (2)</td>
</tr>
<tr>
<td></td>
<td>5119 (2)</td>
</tr>
<tr>
<td></td>
<td><strong>5150±119 (6)</strong></td>
</tr>
<tr>
<td><strong>Human Red Blood Cell</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5159 (2)</td>
</tr>
<tr>
<td></td>
<td>5269 (2)</td>
</tr>
<tr>
<td></td>
<td>5122 (2)</td>
</tr>
<tr>
<td></td>
<td><strong>5184±122 (6)</strong></td>
</tr>
</tbody>
</table>

*a Mean ± SD.
Numbers in parenthesis indicate the number of determinations.

### TABLE 10

**PROPORTIONALITY CONSTANT FOR TISSUE PHOSPHOLIPIDS**

<table>
<thead>
<tr>
<th>Source</th>
<th>cm²/µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human Adrenal</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4883±73 (4)*</td>
</tr>
<tr>
<td></td>
<td>4687 (2)</td>
</tr>
<tr>
<td></td>
<td>5010 (2)</td>
</tr>
<tr>
<td></td>
<td><strong>4860±121 (3)</strong></td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4797±144 (7)</td>
</tr>
<tr>
<td></td>
<td>4814±163 (4)</td>
</tr>
<tr>
<td></td>
<td><strong>4805</strong></td>
</tr>
</tbody>
</table>

*a Mean ± SD.
Numbers in parenthesis indicate the number of determinations.
TABLE 11
TEST OF THE EQUALITY OF TWO VARIANCES

The t-test of the equality of two means requires as one of its assumptions that the population variances are equal. The following method is used to test the equality of the population variance of two independent samples.

Null hypothesis, \( H_0: \sigma_B^2 = \sigma_T^2 \)

Alternative hypothesis, \( H_1: \sigma_B^2 \neq \sigma_T^2 \)

where \( \sigma_B^2 \) and \( \sigma_T^2 \) are the population variance for blood and tissue phospholipids respectively.

Calculations Required

1. Sample variance,

\[
S_B^2 = \frac{n_B \sum_{j=1}^{n_B} x_j^2 - \left( \frac{n_B}{n_B} \sum_{j=1}^{n_B} x_j \right)^2}{n_B(n_B-1)}
\]

\[
S_T^2 = \frac{n_T \sum_{j=1}^{n_T} x_j^2 - \left( \frac{n_T}{n_T} \sum_{j=1}^{n_T} x_j \right)^2}{n_T(n_T-1)}
\]

where \( S_B^2 \) and \( S_T^2 \) are the variances for blood and tissue phospholipids respectively, \( n_B \) and \( n_T \) the number of observations for blood and tissue phospholipids respectively, and \( x_j \) is the individual observations in each set of phospholipids.

2. F statistic for two tail test

\[
F = \frac{\text{larger variance}}{\text{smaller variance}}
\]

Calculations with the Data

1. Sample variance

\[
S_B^2 = 701,708
\]

\[
S_T^2 = 283,334
\]
TABLE 11--Continued.

2. F statistic
\[ F = \frac{701,708}{283,334} = 2.48 \]

3. Two tail test of \( H_0 \)
\[ \sigma_B^2 = \sigma_T^2 \]

<table>
<thead>
<tr>
<th>level of significance</th>
<th>value from F table</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>3.94</td>
</tr>
<tr>
<td>0.10</td>
<td>6.04</td>
</tr>
<tr>
<td>0.02</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Degrees of Freedom: 8,4

Therefore t-test can be used to test the equality of two means.

TABLE 12

TEST OF THE EQUALITY OF TWO MEANS

\[ H_0: PK_B = PK_T \]
\[ H_1: PK_B \neq PK_T \]

where \( PK_B \) and \( PK_T \) are the population means of the proportionality constant for blood and tissue phospholipids respectively.

Calculations Required

1. Samples means

\[
\bar{x}_B = \frac{\sum_{j=1}^{n_B} x_j}{n_B} \quad \bar{x}_T = \frac{\sum_{j=1}^{n_T} x_j}{n_T}
\]

where \( \bar{x}_B \) and \( \bar{x}_T \) are the sample means of the proportionality constant for blood and tissue phospholipids respectively.

2. Sample sum of squares

\[
(n_B-1)S_B^2 = \sum_{j=1}^{n_B} x_j^2 - \left( \frac{n_B}{n_B} \sum_{j=1}^{n_B} x_j \right)^2
\]

\[
(n_T-1)S_T^2 = \sum_{j=1}^{n_T} x_j^2 - \left( \frac{n_T}{n_T} \sum_{j=1}^{n_T} x_j \right)^2
\]

where \((n_B-1)S_B^2\) and \((n_T-1)S_T^2\) are the sample sum of squares for blood and tissue phospholipids respectively.

3. \( t \) statistic

\[
t = \frac{\bar{x}_B - \bar{x}_T}{\sqrt{(n_B-1)S_B^2 + (n_T-1)S_T^2}} \cdot \frac{\sqrt{nBTN(n_B + n_T - 2)}}{\sqrt{n_B + n_T}}
\]

Calculations with the data

The cm²/µmole of all blood and tissue phospholipids samples were used, not their mean values.
TABLE 12—Continued.

1. Sample means
   \[ \bar{x}_B = 5150 \text{ cm}^2/\text{umole} \]
   \[ \bar{x}_T = 4838 \text{ cm}^2/\text{umole} \]

2. Sample sum of squares
   \[ (n_B-1)S_B^2 = 88,489 \]
   \[ (n_T-1)S_T^2 = 56,667 \]

3. t statistic
   \[ t = 5.077 \]

4. Two tail test of \( H_0 \)
   \[ PK_B = PK_T \]

<table>
<thead>
<tr>
<th>level of significance</th>
<th>value from t table</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>1.796</td>
</tr>
<tr>
<td>0.10</td>
<td>2.179</td>
</tr>
<tr>
<td>0.04</td>
<td>2.681</td>
</tr>
<tr>
<td>0.02</td>
<td>3.055</td>
</tr>
</tbody>
</table>

Degrees of Freedom: 12

Reject \( H_0 \) since the t statistic is much larger than \( t_{0.20} \), \( t_{0.10} \), \( t_{0.04} \), and \( t_{0.02} \).

be used to remove glycolipids from nervous and other tissues which contain glycolipids. When column chromatography is used to separate the lipids, as much as 40 column volumes of acetone may be necessary to insure the complete removal of glycolipids.

Preliminary studies in this laboratory suggest that increasing the amount of acetone may decrease the area/µmole for different phospholipids. Thus the proportionality constants for blood and tissue phospholipids may be the same.

The possible explanations for the similarities in the proportionality constant for phospholipids are consistencies in the distribution of molecular species and fatty acid composition from different tissues. Langmuir (15) showed that long chain saturated fatty acids have the same area/molecule which is the cross section area of a methylene group (-CH₂-). Schneider, Holman, and Burr (24) showed that unsaturated fatty acids have nearly the same force-area isotherms. If the surface area is determined by the fatty acid composition, then the ratio of saturated to unsaturated fatty acyl groups indicates the area/µmole for the phospholipids. If the phospholipid molecular species determine the area/µmole, then the fatty acid composition is not a useable parameter to indicate the proportionality

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1R. V. Panganamala, unpublished observations.
constant.

Cornwell, Heikkila, Bar and Biagi (30) reported that the apparent area/molecule for mammalian erythrocyte phospholipids is calculated from the surface area of the total lipid extract.

\[ A'_{PL} = \frac{A_F}{m_{PL}} - \frac{A_{chol} m_{chol}}{m_{PL}} \]  (11)

where \( A'_{PL} \), \( A_F \), and \( A_{chol} \) are the apparent surface area of phospholipid, the surface area of the film, and cholesterol respectively, and \( m_{PL} \) and \( m_{chol} \) are the molecules of phospholipid and cholesterol present in the film. Cornwell et al. (30) assumed that cholesterol and phospholipids are the only lipids present in erythrocytes. The second assumption they made was that the ratio of cholesterol to phospholipid for all mammalian erythrocytes is constant since the condensing effect of cholesterol depends on its molar fraction. The force-area isotherms of the neutral lipid fractions was identical to cholesterol except at low surface pressures. Since the cholesterol/phospholipid ratio for man, rat, bovine, and sheep was nearly the same, the second assumption was also correct. Furthermore Cornwell et al. found that the force-area isotherms for erythrocyte phospholipids from different animals were similar despite the variation in the phospholipid molecular species.

Van Deenen, Houtsmuller, de Haas, and Mulder (135)
studied distearoyl homologues of phosphatidic acid, phosphatidyl ethanolamine, and lecithin. The most stable condensed monolayers of the distearoyl homologues of phosphatidyl ethanolamine and phosphatidic acid had a limiting area (collapse area) of 36Å²/molecule. Distearoyl lecithin under the same conditions had a collapse area of 40Å²/molecule which agreed with the early work of Anderson and Pethica (136).

If the area/molecule is determined by the apolar group, then the limiting area (collapse area) for phosphatidyl ethanolamine and phosphatidic acid is too small. A collapse area of 40Å²/molecule and 44Å²/molecule for distearoyl phosphatidyl ethanolamine and distearoyl lecithin respectively were reported by Phillips and Chapman (137). Thus a limiting area of 20-21Å²/molecule for the stearoyl chain is compatible with the findings of the later investigators. Furthermore van Deenen et al. (135) mentioned that stearoyl oleoyl lecithin had a larger area than stearoyl oleoyl phosphatidyl ethanolamine. Phillips and Chapman attributed the larger area of lecithin to the larger area occupied by the choline group. However, the interpolated area/molecule at 16 dynes/cm of distearoyl lecithin and distearoyl phosphatidyl ethanolamine from both laboratories were 48Å²/molecule and 42Å²/molecule respectively.

Human red blood cells contain approximately 36 percent
lecithin and 28 percent phosphatidyl ethanolamine while human and bovine plasma contain over 70 percent lecithin and 1 to 2 percent phosphatidyl ethanolamine (Table 13). Since the proportionality constant is the same for human and bovine plasma and human erythrocytes phospholipids, the difference in the area/molecule between phosphatidyl ethanolamine and lecithin does not interfere with surface area quantitation.

The effect of the fatty acid composition on the area/molecule of individual naturally occurring phospholipid species was examined by Shimojo and Ohnishi (138). Table 14 gives the fatty acid composition of the various phospholipids species while Table 15 gives the area/molecule at various surface pressures. The limiting area as measured by these authors is the extrapolation of the force-area curves in high pressure to zero pressure. Egg lecithin had a limiting area of $84\text{A}^2$/molecule and contained an equal molar ratio of saturated and unsaturated fatty acids. Phosphatidyl ethanolamine and phosphatidyl serine had smaller limiting areas, $75\text{A}^2$/molecule and $78\text{A}^2$/molecule respectively, but were both 60 percent unsaturated. Similarly the limiting area of sphingomyelin was $68\text{A}^2$/molecule although it was 90 percent saturated. The area/molecule of the phospholipid species is more independent of the fatty acid composition at 10 dynes/cm and 16 dynes/cm. Therefore the difference in the area/molecule between
<table>
<thead>
<tr>
<th>Type</th>
<th>Bovine Plasma</th>
<th>Human Plasma</th>
<th>Human RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin</td>
<td>70.4%</td>
<td>73.0%</td>
<td>34.2%</td>
</tr>
<tr>
<td>Phosphatidyl Choline</td>
<td>0.8</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>5.5</td>
<td>5.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>19.8</td>
<td>16.2</td>
<td>28.4</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>1.9</td>
<td>1.0</td>
<td>13.4</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>1.3</td>
<td>1.0</td>
<td>14.5</td>
</tr>
<tr>
<td>Lyosphosphatidyl ethanolamine</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>2.4</td>
<td>0.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td>1.4</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

Source:


### TABLE 14

**FATTY ACID COMPOSITION OF DIFFERENT PHOSPHOLIPIDS**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Phospholipid</th>
<th>PC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SL&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PS&lt;sup&gt;d&lt;/sup&gt;</th>
<th>PI&lt;sup&gt;e&lt;/sup&gt;</th>
<th>CL&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0&lt;sup&gt;g&lt;/sup&gt;</td>
<td>35.5</td>
<td>28.5</td>
<td>44.5</td>
<td>7.2</td>
<td>4.6</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>--</td>
<td>0.5</td>
<td>--</td>
<td>1.5</td>
<td>--</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>16.1</td>
<td>10.2</td>
<td>4.5</td>
<td>32.8</td>
<td>72.9</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>18:1</td>
<td>32.5</td>
<td>46.5</td>
<td>1.0</td>
<td>54.5</td>
<td>13.6</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>10.5</td>
<td>6.3</td>
<td>--</td>
<td>3.5</td>
<td>4.7</td>
<td>76.5</td>
<td></td>
</tr>
<tr>
<td>18:3</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>20:0</td>
<td>--</td>
<td>trace</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>20:4</td>
<td>5.4</td>
<td>8.0</td>
<td>--</td>
<td>0.5</td>
<td>4.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>22:0</td>
<td>--</td>
<td>--</td>
<td>10.3</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>24:0</td>
<td>--</td>
<td>--</td>
<td>30.2</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>--</td>
<td>--</td>
<td>9.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>% Saturated fatty acids</td>
<td>51.6</td>
<td>38.7</td>
<td>89.4</td>
<td>40.0</td>
<td>77.5</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>% Unsaturated fatty acids</td>
<td>48.4</td>
<td>61.3</td>
<td>10.5</td>
<td>60.0</td>
<td>22.5</td>
<td>99.7</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Phosphatidyl choline (lecithin)

<sup>b</sup>Phosphatidyl ethanolamine

<sup>c</sup>Sphingomyelin

<sup>d</sup>Phosphatidyl serine

<sup>e</sup>Phosphatidyl inositol

<sup>f</sup>Cardiolipin

<sup>g</sup>Number of carbon atoms and number of double bonds.
### TABLE 14—Continued.


### TABLE 15

**AREA \((A^2)\)/MOLECULE OF PHOSPHOLIPIDS AT DIFFERENT SURFACE PRESSURES**

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Limiting Areaa</th>
<th>10 dynes/cmb</th>
<th>16 dynes/cm (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC (d)</td>
<td>84 (A^2)/molecule</td>
<td>80 (A^2)/molecule</td>
<td>78 (A^2)/molecule</td>
</tr>
<tr>
<td>PE</td>
<td>75</td>
<td>71.5</td>
<td>70</td>
</tr>
<tr>
<td>SL</td>
<td>68</td>
<td>66</td>
<td>62</td>
</tr>
<tr>
<td>PS</td>
<td>78</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>PI</td>
<td>85</td>
<td>71</td>
<td>65</td>
</tr>
<tr>
<td>CL</td>
<td>150</td>
<td>150</td>
<td>121</td>
</tr>
</tbody>
</table>

*aShimojo and Ohnishi measured the limiting area by extrapolating the force-area curve in high pressure to zero pressure.

*bObtained from a table in the paper of Shimojo and Ohnishi.

*cInterpolated from force-area isotherms in the paper of Shimojo and Ohnishi.

*dSee Table 14 for abbreviation key.

individual phospholipid species is more dependent on the polar group than the fatty acid composition.

Human and bovine plasma sources have similar phospholipid distribution patterns, while the human red blood cell phospholipids are distributed completely different especially in the percentage of choline containing phospholipids (Table 13). Lecithin accounts for more than 70 percent of the bovine and human plasma phospholipids while an additional 16-20 percent is sphingomyelin. The majority of human red blood cell phospholipids are divided into three groups: lecithin containing 36 percent, sphingomyelin containing 28 percent, and phosphatidyl ethanolamine containing 28 percent. Since the areas/μmole for phospholipids from human plasma and red blood cell and bovine plasma are almost identical, the difference in the type and percentage of the phospholipid species has little significance in determining the proportionality constant of different tissues.

The other proposed explanation for the similarity found for the phospholipid proportionality constants was the fatty acid composition. Van Deenen et al. (135) showed that the area/molecule for films of phosphatidyl ethanolamine and lecithin depend on the chain length and degree of unsaturation of the acyl chains. Shah and Schulman (139) reported that egg and yeast lecithin contained 50 percent and 88 percent unsaturated acyl groups.
Furthermore the increase in the limiting area (collapse area) seen from dipalmitoyl to egg and yeast lecithin was directly proportional to the degree of unsaturation.

The contribution which the fatty acyl chain and the polar sphingosine base make on the area/molecule of two sphingomyelin fractions of ox brain was studied by Raper, Gammack and Sloane-Stanley (140). The first of two fractions isolated contained predominately stearic acid and had a collapse area of 40Å²/molecule. The other fraction contains almost exclusively nervonic acid and had a collapse area of 56Å²/molecule. After hydrogenation of both samples, the molecular area of the stearic acid fraction did not change while the nervonic acid fraction decreased in area to that of the stearic acid fraction. They concluded that the fatty acid portion of the molecule caused the difference in the molecular area between the two sphingomyelin fractions. Furthermore this experiment indicates that the polar end of the long hydrocarbon chain of sphingosine interacts with the subphase. If the double bond of sphingosine was in the air phase, then hydrogenation would have decreased the area of both sphingomyelin fractions.

Shah and Schulman (139) reported that a particular phosphatidial choline contained a saturated, except for the vinyl ether double bond, aldehydogenic chain and an unsaturated fatty acyl chain. Since the fatty acid
composition of this phosphatidal choline is similar to egg lecithin, the force-area isotherms for the two phospholipids should be similar. However, phosphatidal choline has a limiting area (collapse area) of 55Å²/molecule which is 8Å²/molecule smaller than phosphatidylincholine. The difference in the area/molecule was noticed at all surface pressures. Shah and Schulman indicated that the difference was due to the deficiency of one carbonyl group in phosphatidal choline. Therefore it is expected that a tissue with a high content of plasmalogen would have a lower area/molecule. However human erythrocytes which are rich in plasmalogens have the same area/molecule as does human plasma which contains little or no plasmalogens (Table 13). Therefore the fatty acid composition of naturally occurring phospholipids not only controls the area/molecule within each species but determines the area/molecule for the total phospholipid mixture. Thus a likely cause for the similarity is the resemblance in the ratio of saturated to unsaturated fatty acyl groups (Table 16). The small difference in the area/µmole between blood and tissue phospholipids is not the phospholipid distribution patterns. An increase in the amount of saturated fatty acids seems a better explanation of the difference in the proportionality constants. Whatever causes the difference is not as important as recognizing that a difference does exist and quantitating the area of
TABLE 16

FATTY ACID COMPARISON

<table>
<thead>
<tr>
<th>Type</th>
<th>Bovine Plasma\textsuperscript{a}</th>
<th>Human Plasma\textsuperscript{b}</th>
<th>Human RBC\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td>47.7%</td>
<td>49.4%</td>
<td>46.0%</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>52.3%</td>
<td>49.7%</td>
<td>53.0%</td>
</tr>
</tbody>
</table>

Source:

the different phospholipids accordingly.

However, the area/molecule of lysolecithin, which has one fatty acyl chain/molecule, shows no dependence on the fatty acid composition (141). The pressure-area curves for stearoyl and oleoyl lysolecithin were identical which indicates that the oleoyl chain did not expand the area of a saturated lysolecithin film as was seen with lecithin and phosphatidyl ethanolamine films. Furthermore the area of stearoyl lysolecithin and oleoyl lysolecithin were only 15\AA\textsuperscript{2}/molecule less than stearoyl oleoyl lecithin. These two observations indicate that the polar group not the acyl chain controls the area/molecule of lysolecithins. However a decrease in the chain length of the acyl group
or an increase in unsaturation lowers the collapse pressure without changing the area/molecule at lower pressures.

If it is assumed that the fatty acid composition is the major contributor to the area/μmole of naturally occurring phospholipids, then any factors which alter the fatty acid composition can change the molecular area. Two of the prominent determinants of the change in the fatty acid composition are the diet and environmental conditions.

Meyer and Block (142) observed that yeast cell grown anaerobically replaced the unsaturated fatty acids in lecithins with short chain saturated fatty acids. Furthermore these short chain fatty acids, capric, lauric, and myristic, preferred esterification at the β-position of glycerol. Demel, van Deenen, and Pethica (143) found that α-stearoyl β-lauroyl lecithin and α-stearoyl β-oleoyl lecithin had identical pressure area isotherms. Therefore biological active unsaturated acyl chains are replaced in adverse conditions by short chain acyl groups which maintain the physio-chemical properties of the yeast phospholipids.

Law et al. (144, 145) isolated phosphatidyl ethanolamine from E. coli which contained 40 percent C17 and C19 cyclopropane fatty acids and 10 percent unsaturated fatty acids esterified at the β-position of glycerol while the α-position contained straight long chain saturated fatty acyl groups. The cyclopropane containing phosphatidyl
ethanolamines had pressure-area curves which were identical with α-stearoyl β-oleoyl phosphatidyl ethanolamine. Thus the expanded film of phospholipids with unsaturated fatty acids is maintained by different mechanism in yeast and *E. coli*.

Van Golde and van Deenen (146) studied the effects of fatty acid deficient, coconut oil, and corn oil diets on the fatty acid composition and the force-area isotherms of rat liver lecithin. The livers from rats on an essential fatty acid deficient diet, lecithin A, had little linoleic and arachidonic acids but higher levels of eicosatrienoic and oleic acids than rats on coconut oil, lecithin B, or corn oil, lecithin C, diets. When linoleic acid was included in the diet, lecithin B and C, an increase in both linoleic and arachidonic acid was noticed with a decrease in oleic and eicosatrienoic acids. Since the total number of double bonds found in lecithin A was 123, in lecithin B was 132, and in lecithin C was 160, the level of unsaturated fatty acids in rat liver lecithins is controlled. Thus it was not unexpected that the force-area curves of liver lecithins from rats kept on the different diets were nearly identical. Although van Golde and van Deenen (146) compared the number of double bonds to show the similarities in the surface pressure-area isotherms for liver lecithins of rats kept on the three diets, a comparison of the percentage of saturated fatty acids is a better method.
Lecithin A had 44 percent saturated fatty acids, lecithin B had 47 percent, and lecithin C had 43 percent.

Takayasu, Okuda, and Yoshikawa (127) studied the effects of fat free and control diets on the fatty acid composition of rat adrenal phospholipids. Although the percentage of different fatty acids varied between the control and fat free diets, the percentage of saturated and unsaturated fatty acids remained constant.

Dayton, Hashimoto, Dixon, and Pearce (133) studied the effects of prolonged feeding of diets rich in linoleic acid on the serum phospholipids of institutionalized men. The percentage of saturated fatty acids from serum phospholipids did not vary between the linoleate and normal diet after three years. Therefore the percentage of saturated and unsaturated fatty acids is controlled to maintain the physio-chemical properties required for the biological activity of phospholipids. Once the proportionality constant is established for phospholipids for a specific tissue, it should not vary as the diet and environment change.

Verkamp, Mulder, and van Deenen (147) studied the fatty acid composition of phospholipids from various animals. They observed that the fatty acid patterns of phospholipids showed a tissue not the animal specificity that was noticed for the neutral lipids. The tissue specificity was especially obvious in brain and lung
phospholipids from rat, rabbit, pig, horse, ox, and sheep where high levels of palmitic and oleic acid were observed. Furthermore the fatty acid pattern of nuclei, mitochondria, and supernatant cell fractions from rat liver and hepatoma tissues mimic the fatty acid patterns of the whole tissue. Therefore the difference in the fatty acid patterns of phospholipids which Verkamp et al. (147) observed may explain the different proportionality constants observed for tissue and blood phospholipids.

**Standard Curve**

Figure 1 gives the standard curve for cholesterol, triglycerides, blood phospholipids, and tissue phospholipids.
Figure 1. Standard Curve 1. Triglycerides
Statistical Tests on the Proportionality Constants

The one way analysis of variance was used to test the hypothesis that the proportionality constants for triglycerides are the same. Table 17 gives both the calculations required for the test and the analysis of variance table. The F statistic was 0.783 which is much smaller than $F_{0.05}$ or $F_{0.01}$. Therefore the one way analysis of variance shows that the proportionality constants are the same.

The proportionality constants for free cholesterol and saponified cholesteryl esters were compared with the value of 2409 cm$^2$/umole (40A$^2$/molecule) which has been reported previously (29, 136, 144) (Table 18). The t statistic was 1.264 which is smaller than $t_{0.20}$, $t_{0.10}$, and $t_{0.05}$. Therefore the proportionality constant is the same as that reported by other investigators.

The one way analysis of variance was used to test the equality of the proportionality constant for human plasma and erythrocytes and bovine plasma phospholipids (Table 19). The test is the same as that used for the triglyceride proportionality constants. The F statistic was 0.104 which is much smaller than $F_{0.05}$ or $F_{0.01}$. Therefore the proportionality constant for all blood phospholipids are the same.
TABLE 17

STATISTICAL ANALYSIS TO TEST THE EQUALITY OF THE PROPORTIONALITY CONSTANTS FOR TRIGLYCERIDES

$H_0: PK_P - PK_{AG} - PK_{AD} = PK_L$

$H_1: PK_P \neq PK_{AG} \neq PK_{AD} \neq PK_L$

where $PK_P$, $PK_{AG}$, $PK_{AD}$, and $PK_L$ are the proportionality constants for plasma, adrenal, adipose and liver triglycerides respectively.

Calculations Required

1. Correction term, $C$,

$$C = \frac{T^2}{N}$$

where $T$ is the total of all observations and $N$ is the total number of observations.

2. Between sample sum of squares, $SSB$,

$$SSB = \sum_{i=1}^{K} \left( \frac{T_i}{n_i} \right)^2 - C$$

where $K$ is the number of samples, $T_i$ is the total for all observations in each sample, and $n_i$ is the observations in each sample.

3. Total sum of squares, $SST$,

$$SST = \sum_{i=1}^{K} \sum_{j=1}^{n_i} x_{ij}^2 - C$$

where $x_{ij}$ is the individual observations.
TABLE 17--Continued.

4. Error sum of squares, SSE,
   \[ \text{SSE} = \text{SST} - \text{SSB} \]

5. Analysis of variance table

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Samples</td>
<td>( K-1 )</td>
<td>( \text{SSB} )</td>
<td>( \text{MSB} = \frac{\text{SSB}}{K-1} )</td>
</tr>
<tr>
<td>Error</td>
<td>( N-K )</td>
<td>( \text{SSE} )</td>
<td>( \text{MSE} = \frac{\text{SSE}}{N-K} )</td>
</tr>
<tr>
<td>Total</td>
<td>( N-1 )</td>
<td>( \text{SST} )</td>
<td></td>
</tr>
</tbody>
</table>

6. \( F \) statistic

\[ F = \frac{\text{MSB}}{\text{MSE}} \]

Calculations with the Data

1. Analysis of Variance Table with data for triglycerides

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between</td>
<td>3</td>
<td>107,529</td>
<td>35,843</td>
</tr>
<tr>
<td>Within</td>
<td>20</td>
<td>883,087</td>
<td>44,154</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>990,616</td>
<td></td>
</tr>
</tbody>
</table>

2. \( F \) statistic

\[ F = \frac{35,843}{44,154} = 0.783 \]

3. Test of \( H_0 \)

\[ \text{PK}_P = \text{PK}_A = \text{PK}_D = \text{PK}_L \]
TABLE 17—Continued.

<table>
<thead>
<tr>
<th>level of significance</th>
<th>value from F table</th>
</tr>
</thead>
<tbody>
<tr>
<td>.05</td>
<td>3.10</td>
</tr>
<tr>
<td>.01</td>
<td>4.94</td>
</tr>
</tbody>
</table>

Accept $H_0$ since the $F$ statistic is smaller than $F_{0.05}$ or $F_{0.01}$.


TABLE 18

STATISTICAL TEST TO COMPARE CHOLESTEROL WITH THE LITERATURE VALUE OF 2409 cm$^2$/$\mu$MOLE

$H_0$: $PK_{chol} = 2409$ cm$^2$/$\mu$ mole

$H_1$: $PK_{chol} \neq 2409$ cm$^2$/$\mu$ mole

where $PK_{chol}$ is the proportionality constant for free cholesterol and saponified cholesteryl esters.

Calculations with the Data

1. Sample mean, $\bar{x}$,

   $\bar{x} = 2442$

2. Sample variance

   $s^2 = 6132$

3. $t$ statistic

   $t = \frac{\bar{x} - \mu_0}{s \sqrt{n}} = \frac{2442 - 2409}{78} \sqrt{10} = 1.264$

   where $\mu_0$ is the literature value, 2409 cm$^2$/$\mu$ mole for cholesterol
TABLE 18—Continued.

4. Test of \( H_0 \)

\[
PK_{\text{chol}} = 2409 \text{ cm}^2/\mu\text{mole}
\]

<table>
<thead>
<tr>
<th>level of significance</th>
<th>value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>1.383</td>
</tr>
<tr>
<td>0.10</td>
<td>1.833</td>
</tr>
<tr>
<td>0.05</td>
<td>2.262</td>
</tr>
</tbody>
</table>

Accept \( H_0 \) since \( t \) statistic is smaller than \( t_{0.20} \), \( t_{0.10} \), and \( t_{0.05} \).


TABLE 19

STATISTICAL ANALYSIS TO TEST THE EQUALITY OF THE PROPORTIONALITY CONSTANTS FOR BLOOD PHOSPHOLIPIDS

\[
H_0: PK_{\text{BP}} = PK_{\text{HP}} = PK_{\text{HRBC}}
\]

\[
H_1: PK_{\text{BP}} \neq PK_{\text{HP}} \neq PK_{\text{HRBC}}
\]

where \( PK_{\text{BP}} \), \( PK_{\text{HP}} \), and \( PK_{\text{HRBC}} \) are the proportionality constants for bovine plasma, human plasma, and human erythrocytes phospholipids respectively.

Calculations with the Data

1. Analysis of variance table
TABLE 19—Continued.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Samples</td>
<td>2</td>
<td>3,730</td>
<td>1,865</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>432,321</td>
<td>18,013</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>436,051</td>
<td></td>
</tr>
</tbody>
</table>

2. F statistic

\[ F = \frac{1,865}{18,013} = 0.104 \]

3. Test of \( H_0 \)

\( PK_{BP} = PK_{HP} = PK_{HRB} \)

<table>
<thead>
<tr>
<th>level of significance</th>
<th>value from F table</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>3.40</td>
</tr>
<tr>
<td>0.01</td>
<td>5.61</td>
</tr>
</tbody>
</table>

Accept the \( H_0 \) since the F statistic is much lower than \( F_{0.05} \) or \( F_{0.01} \).

The t test was used to compare the similarities between the proportionality constants for liver and adrenal phospholipids. Table 20 gives the values obtained with the data. The t statistic was 1.11 which is lower than \( t_{0.20} \), \( t_{0.10} \), and \( t_{0.05} \). Thus the proportionality constants for liver and adrenal phospholipids are the same.
TABLE 20
STATISTICAL ANALYSIS TO TEST THE EQUALITY OF THE
PROPORTIONALITY CONSTANT FOR TISSUE
PHOSPHOLIPIDS

\[ H_0 : PK_{AD} = PK_L \]
\[ H_1 : PK_{AD} \neq PK_L \]

where \( PK_{AD} \) and \( PK_L \) are the proportionality constants for adrenal and liver phospholipids.

Calculations with the Data

1. Sample mean
\[ \bar{x}_{AD} = 4866 \quad \bar{x}_L = 4800 \]
where \( x_{AD} \) and \( x_L \) are sample means for adrenal and liver phospholipids.

2. Sample variance
\[ S_{AD}^2 = 118,724 \quad S_L^2 = 167,215 \]
where \( S_{AD}^2 \) and \( S_L^2 \) are the sample variance for adrenal and liver phospholipids.

3. \( t \) statistic
\[ t = \frac{\bar{x}_{AD} - \bar{x}_L}{\sqrt{S_{AD}^2 + S_L^2}} \sqrt{\frac{n_{AD}n_L(n_{AD} + n_L - 2)}{n_{AD} + n_L}} = 1.11 \]
where \( n_{AD} \) and \( n_L \) are the number of observations for adrenal and liver phospholipids.

4. Two tail test of \( H_0 \)
\[ PK_{AD} = PK_L \]

<table>
<thead>
<tr>
<th>level of significance</th>
<th>value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.20)</td>
<td>1.33</td>
</tr>
<tr>
<td>(0.10)</td>
<td>1.74</td>
</tr>
<tr>
<td>(0.05)</td>
<td>2.11</td>
</tr>
</tbody>
</table>
TABLE 20—Continued.

degrees of freedom = (n_{AD}+n_{L}-2)-(8+11-2)=17

Accept $H_0$ since statistic is smaller than $t_{0.20}$, $t_{0.10}$ or $t_{0.05}$.

Propagation of Error

The propagation of error determines the percentage which each independent variable contributes to the variance of the proportionality constant. The measurement for the proportionality constant for phospholipids, triglycerides, and cholesterol includes a surface area determination on the sample and colorimetric assays on the sample and standard. The equation used to determine the propagation of error is given below.

$$\frac{\sigma_F^2}{F^2} = \frac{\sigma_a^2}{a^2} + \frac{\sigma_b^2}{b^2} + \frac{\sigma_c^2}{c^2}$$  \hspace{1cm} (12)

where $F$ is the proportionality constant, $a$ the surface area, $b$ and $c$ the optical density of the sample and the standard respectively. $\sigma_F^2$, $\sigma_a^2$, $\sigma_b^2$, and $\sigma_c^2$ are the variance of the proportionality constant, surface area, and colorimetric determinations of the standard and sample respectively.
Table 21 shows that surface area measurements contribute less to the variance than either the colorimetric determination on the standard or sample. Once the proportionality constant is determined for a particular lipid, then the quantitation of that lipid from surface area determinations is more reproducible than from colorimetric determinations.

The surface area and colorimetric determinations on the samples were normalized to compare all values. The absorbance of the phosphorus and cholesterol standard were compared directly since the g/ml of all standards were the same. The triglyceride standard was normalized as absorbance/μequivalent.

**TABLE 21**

<table>
<thead>
<tr>
<th>Lipid</th>
<th>$\frac{\sigma^2_a}{a^2}$</th>
<th>%</th>
<th>$\frac{\sigma^2_b}{b^2}$</th>
<th>%</th>
<th>$\frac{\sigma^2_c}{c^2}$</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.000201</td>
<td>9.2</td>
<td>0.001240</td>
<td>56.5</td>
<td>0.000753</td>
<td>34.3</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.000198</td>
<td>14.9</td>
<td>0.000780</td>
<td>58.6</td>
<td>0.000352</td>
<td>26.5</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.000133</td>
<td>3.1</td>
<td>0.001581</td>
<td>47.1</td>
<td>0.002552</td>
<td>59.8</td>
</tr>
</tbody>
</table>

*a*Surface area measurements.

*b*Colorimetric measurements on the standard.

*c*Colorimetric measurements on the sample.

*d*Values also include cholesteryl esters which were saponified to cholesterol.

Isotope Dilution

The first experiments performed with isotope dilution checked the validity of the method (Table 22). The µmoles of cold cholesterol and triglycerides were determined from surface area measurements using the proportionality constant. Then known aliquots were mixed with 500 µliters of the respective labelled standard. The µmoles of cold cholesterol palmitate were determined gravimetrically and after saponification as the surface area of cholesterol. The labelled and unlabelled cholesteryl palmitate samples were mixed and saponified before area determinations. The amounts of unlabelled sample added to each sample were determined by the isotope dilution method and agreed quite well with the determinations on the cold samples.

The surface area-isotope dilution method was compared to the colorimetric method of Synder and Stephens (67) for the determination of triglycerides. Triglycerides were separated from adipose total lipid in both cases by TLC in 90:10 hexane-diethyl ether (0.5mm Silica Gel H). 500 µliters of labelled triglyceride was added to each sample used in the isotope dilution method before the plates were streaked. The colorimetric assay determined that each sample had 56 µmoles of triglyceride. The surface area isotope dilution method showed 53.6 µmoles of triglyceride present in the sample (Table 23).
## TABLE 22
VALIDITY OF THE ISOTOPE DILUTION METHOD

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>Cholesteryl Palmitate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles of cold cholesterol</td>
<td>µmoles from isotope dilution</td>
<td>% cold cholesterol</td>
</tr>
<tr>
<td>ml cold cholesterol added µmoles</td>
<td>0.715 µmole/ml</td>
<td></td>
<td>0.268 µmole/ml</td>
</tr>
<tr>
<td>5 ml</td>
<td>3.56 µmoles</td>
<td>1.37 µmoles</td>
<td>99.4 %</td>
</tr>
<tr>
<td>10 ml</td>
<td>7.22</td>
<td>2.76</td>
<td>100.8</td>
</tr>
<tr>
<td>10 ml</td>
<td>7.42</td>
<td></td>
<td>103.8</td>
</tr>
</tbody>
</table>

## TABLE 23
COMPARISON OF SURFACE AREA-ISOTOPE DILUTION METHOD WITH COLORIMETRIC ASSAY

<table>
<thead>
<tr>
<th></th>
<th>µmoles of triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorimetric Determination</td>
<td>56.0 µmoles</td>
</tr>
<tr>
<td>Surface Area Isotope Dilution</td>
<td></td>
</tr>
<tr>
<td>Determination A</td>
<td>54.3</td>
</tr>
<tr>
<td>Determination B</td>
<td>53.1</td>
</tr>
</tbody>
</table>
Another isotope dilution experiment combined labelled and unlabelled triglycerides, cholesterol, and cholesteryl palmitate and determined the pmoles of each cold sample (Table 24). The lipids were separated by TLC and after each band was purified, aliquots were taken to determine the surface area and cpm. The amount of each cold lipid added was determined by surface area techniques before mixing. There is very good reproducibility between the cold and isotope diluted samples.

The surface area isotope dilution method quantitated 3.05 pmoles of triglyceride, 6.96 pmoles of cholesterol, and 7.54 pmoles of cholesteryl palmitate. Quantitation of samples much smaller than this is possible by surface area measurements. For example it is possible to quantitate 0.041 pmoles of cholesterol and 0.016 pmoles of triglyceride (Table 25). However the difficulty in such microdeterminations is the quantitative purification of the sample. Thus isotope dilution which requires only the purity not the quantitative recovery of the sample makes such micro-quantitation possible.
TABLE 24
COMBINED STUDY ON CHOLESTEROL, CHOLESTERYL PALMITATE, AND TRIGLYCERIDE

<table>
<thead>
<tr>
<th>Unlabelled Samples</th>
<th>µmoles</th>
<th>Isotope Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>6.96 µmoles</td>
<td>Sample A</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>3.05</td>
<td>µmoles</td>
</tr>
<tr>
<td>Cholesteryl Palmitate</td>
<td>7.54</td>
<td>% unlabelled sample</td>
</tr>
</tbody>
</table>

Sample A
- Cholesterol: 7.00 µmoles
- Triglyceride: 3.25
- Cholesteryl Palmitate: 7.62

Sample B
- Cholesterol: 6.95 µmoles
- Triglyceride: 3.09
- Cholesteryl Palmitate: 7.70

TABLE 25
COMPARISON OF THE LOWER LIMITS OF SURFACE AREA MEASUREMENTS AND ISOTOPE DILUTION

I. Assumption A: Tissue contains only 100cm² of lipid sample.
   - If sample is cholesterol: \( \frac{100}{2442} = 0.040 \mu\text{moles} \)
   - If sample is triglycerides: \( \frac{100}{6410} = 0.015 \mu\text{moles} \)

II. Assumption B: \( S_{\text{STD}} = 1000 \text{ cpm/cm}^2 \)
   
   Prepared by mixing 100,000 cpm of hot compound (\( S = 50 \mu\text{curies/µmole} \) for both cholesterol and tri-palmitin) with 100cm² unlabelled compound.
   
   Placed in 50 ml volumetric flask.
   
   \( S_{\text{STD}} = 1000 \text{ cpm/cm}^2 \)
   
   each ml contains: 2000 cpm
   
   2 cm²
TABLE 25—Continued.

III. Assumption C: Labelled compounds do not contribute to the area (only applies to triglycerides since tripalmitin is hot standard).

\[ S_{\text{tripalmitin}}: 50 \, \text{µcuries/µmole} = 111 \cdot 10^6 \, \text{dpm/µmole} \]

Area of labelled compound:

\[ 100,000 \, \text{dpm} / 111 \cdot 10^6 \, \text{dpm/µmole} = 0.0009 \, \text{µmoles} \]

\[(0.0009 \, \text{µmoles}) \cdot (6410 \, \text{cm}^2/\text{µmole}) = 5.77 \, \text{cm}^2 \]

in 50 ml = 5.77 cm\(^2\)

in 1 ml = 0.11 cm\(^2\)

Therefore area of labelled tripalmitin does not affect the area of triglyceride standard.

IV. Sample

1 ml of hot standard is used for isotope dilution. After sample is extracted, it is diluted to 2 ml with hexane. Then 0.5 ml aliquot is used to obtain the cpm while another 1 ml aliquot is used for surface area measurements.

<table>
<thead>
<tr>
<th>measurement</th>
<th>approximate values expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>area</td>
<td>51 cm(^2/1 , \text{ml} )</td>
</tr>
<tr>
<td>cpm</td>
<td>500 cpm/0.5 ml</td>
</tr>
<tr>
<td>( S_{\text{SAMPLE}} )</td>
<td>( \frac{1000}{51} = 19.6 )</td>
</tr>
</tbody>
</table>

ISOTOPE DILUTION FORMULA:

\[ 100 = 2 \left( \frac{1000}{19.6} - 1 \right) = 102 - 2 \]

V. Conclusion: Therefore the isotope dilution method can be used to lower limits of surface area measurements.
Advantages and Disadvantages

The extreme sensitivity of the method is an important advantage of surface area quantitation over most colorimetric quantitative procedures (Table 26). When the dimensions of the trough are reduced to 50 x 2 x 1 cm, surface area quantitation is 18 to 86 times more sensitive than colorimetric methods used in this study. Table 26 also compares the pmoles required for the lower limits of the surface area measurements and the most sensitive colorimetric methods listed in Table 3. Furthermore the propagation of error statistical analysis showed that the surface area measurements were more reproducible than those from colorimetric determinations.

The time required for the surface area measurements of lipids is about one minute while the triglyceride, cholesterol, and phospholipid colorimetric methods used in this study required two, five, and six hours respectively. Furthermore surface area quantitation eliminates the use of noxious and dangerous chemicals required in some colorimetric procedures. Since surface area measurements involve only the pipetting of the sample and the application of the piston oil, this technique is much simpler than most other methods for the quantitation of lipids.

The major disadvantages of surface area quantitation is the determination of the proportionality constant.
TABLE 26
COMPARISON OF SURFACE AREA AND COLORIMETRIC DETERMINATIONS

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Method</th>
<th>( \mu \text{moles}^a )</th>
<th>Col. ( b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Surface Area</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abell et al. (39)(^c)</td>
<td>0.690</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Zlatkis et al. (53)(^d)</td>
<td>0.155</td>
<td>19</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Surface Area</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lowry et al. (95)(^c)</td>
<td>0.072</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Bartlett (96)(^d)</td>
<td>0.040</td>
<td>10</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Surface Area</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synder and Stephens (66)(^c)</td>
<td>0.220</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Carlson and Wadström (73)(^d)</td>
<td>0.019</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\) \( \mu \)Moles required to give lower limit values.

\(^b\) The magnitude of the difference in the sensitivity of the surface area and colorimetric methods.

\(^c\) Methods used in this study.

\(^d\) Most sensitive method listed in Table 3.
A method for the microdetermination of free and esterified cholesterol, phospholipids, and triglycerides from surface area measurements is described. The area occupied by lipids and other surface area occupying compounds is directly proportional to the amount of material added.

\[ A = K \mu M \]

where \( A \) is the surface area in cm\(^2\), \( K \) is the proportionality constant, cm\(^2\)/\( \mu \)mole, and \( \mu M \) is the \( \mu \)moles of material added.

The proportionality constant for triglycerides from adipose, adrenal, liver, and plasma was 6511 cm\(^2\)/\( \mu \)mole. The proportionality constant for blood phospholipids, human erythrocytes and plasma and bovine plasma, and tissue phospholipids, human adrenal and liver were 5150 cm\(^2\)/\( \mu \)mole and 4838 cm\(^2\)/\( \mu \)mole respectively. Cholesterol and saponified cholesteryl esters had a proportionality constant of 2442 cm\(^2\)/\( \mu \)mole. The cholesteryl esters were saponified since long chain esters of cholesterol are too nonpolar to form stable monolayers.
Langmuir (15) showed that long chain saturated fatty acids have identical force-area curves while Schneider et al. (24) reported that unsaturated long chain fatty acids have similar force-area curves. Thus the probable cause for the similarities in the proportionality constants for phospholipids and triglycerides from different tissues is the resemblance seen in the percentage of saturated and unsaturated fatty acids of these lipids. The phospholipid molecular species distribution was eliminated as a cause for the similarity in the proportionality constants. Human and plasma phospholipids have completely different phospholipid distribution patterns but their proportionality constants were nearly identical.

If the dimensions of the trough are 50 x 2 x 1 cm, surface areas as small as $20 \text{ cm}^2$ are sufficient to quantitate lipids. This area corresponds to 0.008, 0.003, and 0.004 μmoles of cholesterol, triglycerides, and phospholipids. The quantitative purification of such small samples posed a major problem that was eliminated by isotope dilution which requires only the purification not the quantitative recovery of the sample.

The measurement of surface area requires only the pipetting of the sample and the application of the piston oil. However, the purity of each lipid sample is required since contamination of the sample with lipids or other surface active materials causes inaccurate results.
The time required for surface area quantitation is about one minute/determination compared to several hours required for colorimetric determinations.

The propagation of error statistical analysis showed that the surface area measurements contributed less to the total variance than either the colorimetric assay on the sample or standard. Once the proportionality constant is established for lipids, then quantitation by surface area measurements are more reproducible, sensitive, and rapid than colorimetric methods. However, the proportionality constants of phospholipids and triglycerides from each new tissue must be determined before that lipid can be quantitated. Furthermore, the proportionality constants for all lipids must be routinely checked to insure the accuracy of surface area quantitations.
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