Surface areas of naturally occurring lipid classes and the quantitative microdetermination of lipids

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Abstract The surface area (A) of a lipid was directly proportional to the amount of lipid in a surface film ($A = k$ µmoles) measured at constant surface pressure, temperature, and subphase composition. A surface area of 2300 cm²/µmole was obtained for cholesterol isolated from human adrenal and aorta and for cholesterol from hydrolysates of cholesteryl esters isolated from the same tissues. Unsaturated methyl esters that contained from one to four *cis* double bonds had the same surface area, 39.4 Å²/molecule. As a consequence, naturally occurring triglyceride mixtures which had similar saturated–unsaturated fatty acid ratios had the same surface area, 6090 cm²/µmole. Naturally occurring phospholipid mixtures had the same surface area, 4590 cm²/µmole, and it appeared that the composition of these mixtures was regulated to control the physical properties of the mixtures. Surface area was much more sensitive than colorimetric procedures for the estimation of cholesterol and triglycerides. The surface area/molecule was a criterion of purity and an expanded surface area/molecule was an indication of autoxidation. Thus, surface area measurements were valuable for both the microdetermination and the characterization of lipid classes.

Supplementary key words Langmuir trough · piston oils · cholesterol · cholesteryl esters · methyl esters · triglycerides · phospholipids · area/molecule · area/hydrocarbon chain · film stability · colorimetry · physical properties of mixtures

SURFACE-ACTIVE compounds occupy large areas as monomolecular films. At constant surface pressure, temperature, and subphase composition, the surface area (A) of a pure compound is directly proportional to the number of molecules in the surface:

$$A = k \ \mu\text{moles} \qquad \text{Eq. 1}$$

where k is a proportionality constant that depends on the structure of the compound (1). In the present investigation, we have developed a surface area technique based on this relationship (Eq. 1) for the microdetermination of cholesterol.

Although naturally occurring triglycerides are complex mixtures which contain several fatty acids, the relative amounts of total saturated and total unsaturated fatty acids do not vary widely in the triglyceride mixtures isolated from different individuals or from different tissues in the same individual unless the dietary regimen is drastically altered (2). The major saturated fatty acids of human triglycerides, palmitic and stearic acids, have the same surface area (3). Oleic acid has a much larger surface area than the saturated fatty acids (3), but early studies suggested that the introduction of additional *cis* double bonds did not cause comparable increments in surface area (4, 5). These observations, a reasonably constant saturated–unsaturated fatty acid ratio and similarities in the surface areas of all unsaturated fatty acids, suggested that even complex mixtures such as the naturally occurring triglycerides could be quantified from surface area measurements. In the present investigation, we have measured the surface areas of the methyl ester and triglyceride derivatives of pure fatty acids, and we have developed a surface area technique for the microdetermination of naturally occurring triglyceride mixtures.

The surface area of a phospholipid depends on both its nitrogen base and its fatty acid components. Choline phosphoglycerides have larger surface areas than

Abbreviations: TTP, tri-*m*-tolyl phosphate; BHT, 2,6-di-*tert*-butyl-4-methylphenol; TLC, thin-layer chromatography.

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ethanolamine phosphoglycerides with the same fatty acid composition (6–9). Phospholipids containing unsaturated fatty acids have larger surface areas than phospholipids containing saturated fatty acids (6, 7). Furthermore, the surface area of a phospholipid is not a simple function of the saturated–unsaturated fatty acid ratio. For example, 1-stearoyl-2-oleoyl and 1,2-dioleoyl choline phosphoglycerides have similar surface areas which are much smaller than the surface area of 1,2-dilinoleoyl choline phosphoglyceride (7). Nevertheless, Cornwell et al. (10) found that erythrocyte phospholipid mixtures from different animal species had similar mean surface areas even though the phospholipid mixtures varied widely in nitrogen base and fatty acid compositions. These investigators used the mean surface area for the phospholipids from human erythrocytes and the phospholipid content of erythrocytes from other species to approximate the surface areas of the erythrocytes from these species. This observation, a characteristic mean surface area for several naturally occurring phospholipid mixtures, suggested that phospholipid mixtures could be quantified from surface area measurements. In the present investigation, we have developed a surface area technique for the microdetermination of naturally occurring phospholipid mixtures.

MATERIALS AND METHODS

Analytical grade solvents were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. Anhydrous ether was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. Commercial *n*-hexane was purified as previously described (11). All solvents were redistilled and all solvents except acetone were passed through a basic alumina (Woelm Basic, activity grade 1) column employing 500 g of alumina/3 l of solvent. Cholesterol, cholesteryl oleate, methyl palmitate, methyl oleate, methyl arachidonate, triolein, trilinolein, and trilinolenin were purchased from Applied Science Laboratories, State College, Pa. Methyl linoleate, methyl linolenate, and tripalmitin were purchased from the Hormel Institute, Austin, Minn. A primary cholesterol standard was obtained from the National Bureau of Standards, Washington, D.C. Castor oil was purchased from E. R. Squibb & Sons, New York. Tri-*m*-tolyl phosphate (TTP) was purchased from Eastman Organic Chemicals, Rochester, N.Y. The antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT) was purchased from Aldrich Chemical Co., Milwaukee, Wis. Blood was collected in acid–citrate–dextrose from healthy volunteers. Human adrenals, aorta, perirenal fat, brain, and liver were obtained at autopsy and the tissues were stored at −20°C until used.

Extraction and purification of lipids

Plasma and erythrocytes were separated by centrifugation. Erythrocyte lipids were extracted by the method of Rose and Oklander (12), and lipids were extracted from plasma and tissues as previously described (13). All solvents used in extractions contained 0.001% BHT. The total lipid mixture was placed on a Unisil column. Neutral lipids were eluted with 4 column volumes of chloroform, glycolipids were eluted with either 8 or 40 column volumes of acetone, and phospholipids were eluted with 4 column volumes of methanol. The phospholipid fraction was monitored for glycolipids and the glycolipid fraction was monitored for phospholipids by TLC as previously described (13). The glycolipid fraction was also monitored for lipid phosphorus.

The neutral lipid fraction was streaked on a TLC plate coated with 0.25 mm or 0.5 mm of silica gel H and predeveloped with chloroform–methanol 2:1 (v/v). The plate was developed with hexane–ether 90:10, giving cholesteryl ester (R_F 0.8), triglyceride (R_F 0.5), and cholesterol (R_F 0.1) bands. Lipid fractions were eluted with ether. When samples were rich in cholesteryl esters, triglycerides were purified on a second TLC plate which was developed with hexane–ether 90:10. Diglycerides and free fatty acids were removed from cholesterol fractions by saponification and extraction of nonsaponifiable cholesterol with hexane (14). Cholesterol was further purified by TLC using a chloroform–acetone 90:10 solvent system (15). In an alternate procedure, the neutral lipid fraction was streaked on a TLC plate and developed with chloroform–acetone 90:10, giving a cholesteryl ester–triglyceride band (solvent front) and a cholesterol band (R_F 0.64). Cholesteryl esters and triglycerides were then separated on a second TLC plate developed with hexane–ether 90:10 as described above.

Because Kwong, Heikkila, and Cornwell (16) showed that cholesteryl esters did not form stable monolayers, the cholesteryl esters were converted to cholesterol and methyl esters with sodium methoxide (17). The solution was neutralized with aqueous hydrochloric acid, extracted with hexane, and concentrated. The reaction products were placed on a TLC plate and the plate was developed with chloroform–acetone 90:10. Cholesterol (R_F 0.64), which was separated from methyl esters (solvent front) and free fatty acids (R_F 0.36), was extracted with ether. In an alternate procedure, cholesteryl esters were first saponified by the procedure of Brooks et al. (18), and an aliquot of the nonsaponifiable material was streaked on a TLC plate which was then developed with chloroform–acetone 90:10. Concentrated cholesteryl ester fractions were not completely saponified by the procedure of Abell et al. (14).

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Standard colorimetric methods were used for the estimation of lipid phosphorus (19), fatty acid esters (20), and cholesterol (14).

Surface area

Constant pressure–variable area measurements were obtained with a floating barrier and piston oils on 10 × 50 × 1 cm or 2 × 50 × 1 cm Teflon troughs as previously described (21). The subphase contained 0.02 M sodium chloride.

Castor oil, which generated 17 ± 0.7 dynes/cm, was used as the piston oil for cholesterol and phospholipids. Because equilibrium spreading pressures for triglycerides were near 12 dynes/cm (16), TTP, which generated 9.5 dynes/cm, was used as the piston oil with this lipid fraction. Surface area was measured 2 min after applying the piston oil, at which time the barrier had been compressed to a stationary position. The temperature was maintained at 24°C.

The surface was cleaned by suction after each measurement and the trough was cleaned periodically with a potassium dichromate–sulfuric acid solution. A clean surface was assumed when the moving barrier was moved to either end of the trough and remained stationary at these positions.

Highly purified lipid solvents sometimes contained trace amounts of nonvolatile components which contributed to the surface area. Reagent blanks were carried through the extraction and purification procedures used for the isolation of triglyceride, phospholipid, cholesterol, and cholesteryl ester fractions. Sections of the same TLC plate were used for both blank and sample. When reagent blanks were diluted to a final volume of 2 ml, 100-μl aliquots generated 8.5 ± 3.2 cm² on the Teflon trough. A reagent blank was required when lipids were estimated in very dilute solutions.

RESULTS AND DISCUSSION

Cholesterol and cholesteryl esters

Known amounts of cholesterol were spread on the trough and the proportionality constant for cholesterol, k_c (Eq. 1), was determined. This proportionality constant was the same for cholesterol standards, free cholesterol isolated from adrenal and aorta, and cholesterol isolated from adrenal and aorta cholesteryl ester hydrolysates (Table 1). The surface area of the cholesterol film was unchanged after 45 min.

The area/molecule of cholesterol, 38.2 Å² (Table 1), was identical with the area/molecule reported by Adam (22). Cholesterol and cholesteryl ester fractions from human tissues sometimes contain trace amounts of cholesterol oxidation products such as 26-hydroxycholesterol, 7β- and 7α-hydroxycholesterol, and 24-hydroxycholesterol (18), and these compounds have larger surface areas than cholesterol (15). For example, cholesterol isolated from erythrocytes had a larger surface area than 38 Å²/molecule (10). In the present study, cholesterol samples were separated from oxidation products by the TLC procedure of Kamel, Weiner, and Felmeister (15). Thus, it appeared that the k_c value was useful as one criterion of cholesterol purity.

TABLE 1. Proportionality constant for cholesterol from cholesterol standards and cholesterol isolated from several human tissues measured at constant surface pressure using castor oil as the piston oil

Source[a]		k_c Sample	k_c Mean ± SD
		cm²/μmole	
Standard			
AS		2260	
		2310	2280 ± 22
NB		2270	
		2280	
Free Cholesterol			
Adrenal	A	2260	
		2230	
	B	2350	
		2250	2300 ± 72
Aorta	A	2280	
		2430	
		2230	
		2350	
Cholesteryl esters			
Adrenal	A	2270	
		2240	
	B	2250	
		2400	2310 ± 71
		2380	
Aorta	A	2380	
		2260	
Mean ± SD[b]		2300 ± 69[c]	

[a] Standards were purchased from Applied Science Laboratories (AS) and the National Bureau of Standards (NB). Tissue samples are designated by a letter. Triplicate surface area and cholesterol measurements were used for each analysis. Cholesteryl esters were hydrolyzed before the surface area measurement (see text).
[b] Mean ± SD for free cholesterol and cholesteryl ester samples from human tissue.
[c] Equivalent to 38.2 ± 1.1 Å²/molecule.

Methyl esters and triglycerides

The area/molecule of pure methyl esters and triglycerides was measured because the feasibility of a surface area technique for the microdetermination of naturally occurring triglycerides depended on the assumption that all *cis* unsaturated fatty acids had similar surface areas. Methyl esters were chosen because unsaturated fatty acids are quite soluble and desorb rapidly from surface films (21). Unsaturated methyl esters that contained from one to four double bonds had the same surface area (Table 2). These data extended the work of Adam and Dyer (4) and Schneider, Holman, and Burr

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TABLE 2. Surface areas for pure methyl esters and triglycerides measured at constant surface pressure using TTP as the piston oil

Lipid	Surface Area: Molecule	Surface Area: Hydrocarbon Chain
	Å^2	
Palmitic acid[a]	22.8 ± 0.4[b]	22.8
Methyl oleate (3)[c]	39.0 ± 0.4	39.0
Methyl linoleate (2)	39.7	39.7
Methyl linolenate (4)	39.2 ± 0.6	39.2
Methyl arachidonate (3)	39.8 ± 0.8	39.8
Tripalmitin (1)	62.0	20.7
Triolein (4)	102.2 ± 1.6	34.1
Trilinolein (5)	108.9 ± 0.6	36.3
Trilinolenin (2)	108.2	36.1

[a] Data from Heikkila, Kwong, and Cornwell (11) at 10 dynes/cm.
[b] Means ± SD.
[c] Number of lipid samples weighed is in parentheses. Surface area of each sample was measured at least three times.

TABLE 3. Proportionality constant for triglycerides isolated from several human tissues measured at constant surface pressure using TTP as the piston oil

Tissue[a]		k_{tg}: Sample	k_{tg}: Mean ± SD
		cm²/µmole	
Adipose	A	6040	
	B	6090	6030
	C	5970	
Adrenal	A[b]	6460	
	B	6230	
	C	5960	
	D	5970	6150 ± 204
	E	6280	
	F	6010	
Aorta	A[b]	5930	
	B	6010	6020 ± 79
	C	6030	
	D	6120	
Liver	A	6180	
	B	5980	6080
Plasma	A	6240	
Mean ± SD		6090 ± 147[c]	

[a] Each tissue is designated by a letter. Surface area and ester measurements were made in triplicate for each analysis.
[b] Triglycerides were purified twice by TLC.
[c] Equivalent to 101 ± 2.4 Å^2/molecule or 33.7 ± 0.8 Å^2/hydrocarbon chain.

(5), who found similar but not identical surface areas for several unsaturated fatty acids. Schneider et al. (5) showed a significantly expanded arachidonic acid film, but their data probably reflected film expansion through sample oxidation (16). Surface areas of methyl ester films decreased from 1 to 2% in 5 min. The methyl palmitate film decreased 6% in 45 min while the methyl linolenate film decreased 25% in the same time interval. Gaines (3) reported a 10% decrease in the area of a methyl oleate film in 30 min. The decrease in film area apparently varied directly with the solubility of the compound.

Pure unsaturated triglycerides had similar surface areas (Table 2). The area/hydrocarbon chain in these symmetrical triglycerides was lower than the area/hydrocarbon chain in the corresponding methyl esters (Table 2). Similar data were reported by Gaines (3) for methyl oleate and triolein. These data suggested highly specific packing modes for pure symmetrical triglycerides, and indeed Merker and Daubert (23) found with mixed triglycerides that the more symmetrical compounds had smaller surface areas. Triglyceride films were stable. For example, the surface area of trilinolein decreased only 4% in 25 min.

Naturally occurring triglycerides purified from several human tissues were similar in fatty acid composition, and these triglycerides all had the same proportionality constant. Triglycerides isolated from adrenal and aorta, tissues that contain large amounts of other neutral lipids, required two TLC separation steps in their purification (Table 3). Naturally occurring triglycerides formed stable films. For example, the surface area of triglycerides isolated from human aorta decreased only 1 to 2% in 45 min. Films showed no evidence of the expansion which is characteristic of the initial oxidation reactions in monolayers (16, 24). Merker and Daubert (25) previously noted that oxidation was not a significant problem with films of unsaturated monoglycerides.

The mean area/hydrocarbon chain, A_m, in naturally occurring triglycerides was 33.7 ± 0.8 Å^2 (Table 3). If the surface film technique is valid, this area should be approximated by the equation:

$$A_sR_s + A_uR_u = A_m \quad \text{Eq. 2}$$

where A_s and A_u are the areas of saturated and unsaturated chains and R_s and R_u are the relative molar concentrations of saturated and unsaturated fatty acids in the naturally occurring triglycerides. For unsymmetrical triglycerides, A_s and A_u are undoubtedly more nearly approximated from fatty acid and methyl ester data, 22.8 and 39.4 Å^2, respectively (Table 2), than from symmetrical triglyceride data. Triglycerides isolated from adipose, adrenal, aorta, plasma, and liver tissue of three individuals contained 35.9 ± 5.3 (mean ± SD) weight % saturated fatty acids (2) or 38 ± 6 mole % saturated fatty acids. When these area and composition data were substituted in Eq. 2, the calculated A_m was 33.1 Å^2 and the range in A_m was 32.1–34.1 Å^2. Thus, calculated and experimental A_m data were in reasonable agreement, and it was apparent that a surface film technique was suitable for quantitative studies on the naturally occurring triglycerides from several human tissues.

Phospholipids

Proportionality constants, k_p, for phospholipids isolated from several human tissues are summarized in

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TABLE 4. Proportionality constants for phospholipid mixtures isolated from several human tissues measured at constant surface pressure using castor oil as the piston oil

Tissue[a]		k_p[b]			
		I		II	
		Sample	Mean ± SD	Sample	Mean ± SD
		cm²/µmole			
Adrenal	A	5480		4900	
		4700		4690	
	B	4860	4780 ±	4740	4660 ±
		4620	320	4530	130
		4620		4540	
	C	4620		4640	
		4590		4590	
Aorta	A	4570		4400	
		4560		4480	
	B	4830		4460	
		4810	4880 ±	4340	4510 ±
	C	5570	454	4630	96
		5620		4670	
	D	4580		4590	
		4530		4480	
	E			4540	
				4520	
				4500	
Brain	A	5450		4760	
		4990	5090	4470	4490 ±
	B	4840		4360	189
				4360	
Liver	A	4880		4720	
	B			4900	4710 ±
		4620	4800	4600	165
				4490	
	C	4900		4820	
Plasma	A	4690		4720	4640
				4560	
Erythrocyte	A	4650		4630	4710
				4800	
Mean ± SD		4850 ± 344		4590 ± 151[c]	

[a] The total lipid extract from one tissue is designated by a letter. Each sample represents phospholipids isolated from an aliquot of the designated total lipid extract. Triplicate surface area and quadruplicate phosphorus measurements were used in a sample analysis.

[b] Glycolipids were eluted from the Unisil column with either 8 column volumes of acetone (I) or 40 column volumes of acetone (II) prior to phospholipid elution with methanol.

[c] Equivalent to 76.2 ± 2.5 Å²/molecule or 38.1 ± 1.2 Å²/hydrocarbon chain.

Table 4. Phospholipids were separated from glycolipids by two procedures. In the first procedure, the glycolipids were eluted with 8 column volumes of acetone. High k_p values were sometimes obtained, particularly with brain, and the standard deviation was large (see column I in Table 4). In the second procedure, the glycolipids were eluted with 40 column volumes of acetone. Reproducibility was improved by the second procedure, and there was little difference between the mean k_p value calculated for all phospholipids and the k_p value for a specific tissue (see column II in Table 4). The glycolipids which were eluted with acetone formed surface films, and preliminary experiments indicated that glycolipids could also be estimated from their surface areas. In fact, larger surface areas for erythrocyte phospholipid mixtures were reported by Cornwell et al. (10) when glycolipids were not separated from the glycolipid–phospholipid fraction.

Naturally occurring phospholipid mixtures formed stable films which decreased only 1–2% in area in 45 min. Thus, phospholipid films behaved like cholesterol and triglyceride films and were not oxidized in a 45-min interval. We added BHT to lipid solvents to prevent lipid oxidation during extraction and separation procedures. Films of partially oxidized lipid are expanded (16, 24) and as a consequence the surface area/molecule is a sensitive indicator of lipid oxidation.

The mean surface area for phospholipid mixtures, 76.2 ± 2.5 Å²/molecule (Table 4), was within the range in surface areas reported for specific phospholipids isolated from egg yolk and bovine tissues (8). The mean surface area for phospholipid mixtures (Table 4) was very similar to the mean surface area for phospholipid mixtures isolated from the livers of rats maintained on fat-free and coconut oil diets (7). Indeed, Demel (7) has suggested that the fatty acid composition of membrane phospholipids is regulated to preserve the physical properties of these lipids. We suggest that both fatty acid and nitrogen base compositions are regulated to preserve the physical properties of phospholipid mixtures. Consequently, the area/molecule is the same for different naturally occurring phospholipid mixtures, and surface area may be used for the quantitative estimation of naturally occurring phospholipid mixtures. Membrane permeability is altered by a change in cholesterol content (26). Rothman and Engelman (27) have suggested recently that cholesterol interacts with both saturated and unsaturated acyl chains in phospholipids. Thus, it is possible that the cholesterol content regulates in part the membrane properties of phospholipid mixtures which differ in composition but have very similar physical properties. In an analogous manner, glycolipids may alter the membrane properties of the phospholipid mixtures in erythrocytes and myelin.

Comparison of surface area and colorimetric procedures for the estimation of lipid classes

Surface area provides a sensitive procedure for the determination of lipid classes. Because the surface film procedure requires the separation of lipid classes, total cholesterol and lipid phosphorus in a lipid extract are more readily measured by colorimetric procedures. However, it is sometimes necessary to separate lipid classes even when colorimetric procedures are employed. Thus, cholesteryl esters must be separated from cholesterol prior to their estimation by a colorimetric procedure. Furthermore, the total sample rather than an

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aliquot may be needed with small amounts of lipid for the estimation of one lipid class, and as a consequence the mixture must be fractionated before each lipid class is estimated by a colorimetric procedure.

The surface film procedure requires a reagent blank which is carried through all separation and isolation steps when lipids are determined in micro amounts. Nevertheless, sensitivity is the major advantage of this technique over colorimetric techniques for the quantitation of lipid classes. Surface area and colorimetric procedures are compared in Table 5. The comparisons are based on the amount of lipid generating 40 cm^2 and the amount of lipid generating 0.200 absorbance units since these quantities are sufficient for at least duplicate analyses. Surface area is much more sensitive than absorbance for the estimation of triglycerides and cholesterol and is somewhat more sensitive than absorbance for the estimation of lipid phosphorus. In a preliminary study involving lipids from individual lesions in human coronary arteries, we have found that total lipid often weighed less than 0.5 mg and surface area was necessary for the estimation of triglycerides, cholesteryl esters, and cholesterol. These data will be reported in a subsequent study.

When the area/molecule for a lipid class is established and sufficient lipid is available for both surface area and colorimetric measurements, the area/molecule is one important criterion of purity for the lipid class. With small samples, the area/molecule for cholesterol is more readily determined than its melting point. The separation of glycolipids and phospholipids may be monitored by measuring surface area in fractions eluted from a chromatographic column. In fact, surface area provides a rapid and sensitive indication of lipid in column eluates. Lipid mixtures such as triglycerides or phospholipids may be evaluated for autoxidation by measuring changes in area/molecule. Thus, surface area measurements are valuable both for the microdetermination and for the further characterization of lipid classes.

TABLE 5. Lipid required for the estimation of lipid classes by surface area and colorimetric procedures

Lipid Class	Lipid[a]		Absorbance/ Surface Area
	Surface Area[b]	Absorbance[c]	
	mg		
Triglyceride	0.0058	0.19	33
Cholesteryl ester	0.011	0.50	45
Cholesterol	0.0066	0.30	45
Phospholipid	0.0067	0.059	8.8

[a] Calculated assuming molecular weights of 885 for triglyceride, 651 for cholesteryl ester, and 775 for phospholipid (4% phosphorus).
[b] Amount of lipid generating 40 cm^2 as a surface film.
[c] Amount of lipid generating 0.200 absorbance units as fatty acid ester (20), cholesterol (14), or lipid phosphorus (19).

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