Chain-Substituted Lipids in Monolayer Films. A Study of Molecular Packing

F. M. Menger,* M. G. Wood, Jr., S. Richardson, Q. Zhou, A. R. Elrington, and M. J. Sherrod

Contribution from the Department of Chemistry, Emory University, Atlanta, Georgia 30322.
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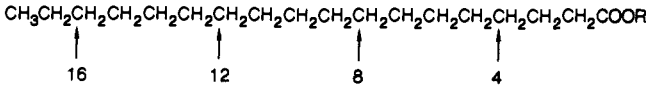
Abstract: A series of highly purified fatty acids and phospholipids, each possessing a chain substituent of varying size (methyl, *n*-butyl, or phenyl) at varying locations (carbon 4, 8, 12, or 16 of an 18-carbon chain) were synthesized. Pressure–area isotherms, obtained with the aid of a film balance, revealed how these molecules, either individually or admixed, pack in monomolecular films. Two examples will illustrate here the type of information secured by the method. (a) The presence of a methyl at carbon 16 of a single phospholipid chain has a negligible effect on the molecular packing within a "liquid" film. A methyl on carbon 16 of both chains, on the other hand, is highly expansive. Even a single methyl at position 8 perturbs the film packing appreciably, thus indicating less available space near the center of the chain. (b) Deviations from ideality in mixed fatty acid films can be explained by interdigitation that reduces steric repulsion among the substituents. It is also possible to observe by means of film balance techniques the extrusion of 8-butylstearic acid when it contaminates a film composed largely of stearic acid. Approximate SHADOW calculations could, in many cases, provide film areas in reasonable agreement with experiment.

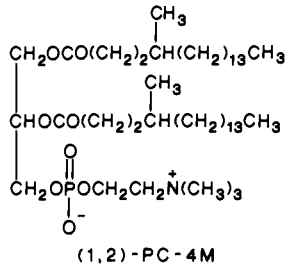
There is a growing need for chemists, particularly those with biological leanings, to understand the principles governing noncovalent interactions. Host and guest, sensor and activator, channel and permeant, receptor and drug, enzyme and substrate, antibody and antigen, DNA and carcinogen: all partners recognize each other by means of noncovalent forces. Since intermolecular association and organization is involved in so many vital processes, we initiated a study of "molecular packing" using lipids as the main focus. Three weak brothers of covalency (hydrogen bonding, hydrophobic attraction, and electrostatics) assemble lipids into a community of molecules. As with human communities, the individual species dictate the behavior of the group while, concurrently, the group imposes constraints upon the individuals.[1] Our goal is to understand this interrelationship in greater detail.

Phospholipids possess two hydrophobic chains attached to a hydrophilic head-group. When exposed to water, many phospholipids spontaneously form molecular bilayers. The chains point inwardly side-by-side, whereas the head-groups reside on the surfaces of the sheet. Most biological membranes are constructed of such phospholipid bilayers. Their properties, including permeability, depend upon "packing" which, in turn, depends upon lipid structure. For example, a high degree of unsaturation along the chains increases permeability[2] because the double bonds impair a tight association among the hydrocarbon tails. In the work described below, we examine synthetic fatty acids and phospholipids composed of 18-carbon chains substituted at one of four loci with various groups. Both chains of the phospholipids, or

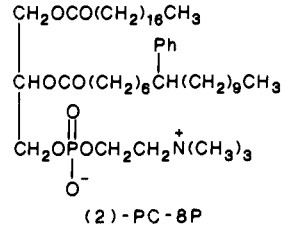


only chain 2, were modified in this manner. Owing to the large number of compounds, it is necessary to adopt a shorthand notation. Thus, when both octadecanoyl chains possess a methyl group at C_4, the phosphatidylcholine will be designated (1,2)-PC-4M. And a lipid having a C_8 phenyl group on only the second chain will be called (2)-PC-8P.

Packing properties of the chain-modified lipids were examined by means of a particularly simple and ingenious device: the film balance.[3,4] A film balance measures the pressure exerted by a film, one molecule thick, resting on a water surface. By adjusting a movable barrier, the area available to the film can be diminished.



(1,2)-PC-4M



(2)-PC-8P

Molecules lying flat on the water surface are thereby forced into more vertical positions where they require less space. The ease of compressing the film, and the area per molecule at a given pressure, reveal how readily a lipid molecule tolerates the presence of neighbors. One might presume, for example, that a phenyl group residing on a lipid chain should lead to more "expanded" films. This was found to be the case. Just how the perturbation depends upon the number, location, and size of the substituent forms the basis of the present paper. Since a monomolecular film can be regarded as "half a bilayer", the data pertain directly to membrane behavior.

Early work on the packing of substituted fatty acids was reported by Izawa in Japan,[5] Stenhagen in Sweden,[6] and Weitzel in Germany.[7] More recently, Nagarajan in India,[8] Tachibana in Japan,[9] and Zografi and Cadenhead in the United States[10] have published pressure–area isotherms of hydroxy- and keto-substituted fatty acids. Monolayers of four terminally branched phospholipids have been examined by Kannenberg and Cadenhead.[11] To our knowledge, systematic investigations of molecular packing within films of chain-substituted phospholipids have never been carried out. Our film balance studies on such systems, embellished by differential scanning calorimetry and molecular mechanics SHADOW calculations, are described below.

Experimental Section

Synthesis. Since the synthesis of all alkyl- and aryl-substituted fatty acids, and phospholipids derived from them, followed the same route

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Table I. Yields for the Syntheses of *dl* Branched Octadecanoic Acids[a,b]

position	yield, %					
	a	b,c	d	e	f	g
8-Bu	73	47	90	92	83	90
8-Me			85	78	86	91
8-Ph			55	76	83	87
12-Bu	58	67	94	98	84	76
12-Me			95	64	94	94
12-Ph			99	80	86	77
16-Me	68	70	92	62	88	88

[a] Letters a–g refer to reactions in Figure 1. [b] For the synthesis of 4-methyloctadecanoic acid and 4-butyloctadecanoic acid, another procedure was followed: Cason, J.; Wolfhagen, A. J.; Tarpey, W.; Adams, R. E. *J. Org. Chem.* **1949**, *14*, 147.

Table II. Yields for the Syntheses of Branched-Chain Phosphatidylcholines[a]

name	yield, %		name	yield, %	
	a	b		a	b
(1,2)-PC-4M	72		(1,2)-PC-12M	75	
(2)-PC-4M		42	(2)-PC-12M		51
(1,2)-PC-8M	81		(1,2)-PC-12P	71	
(2)-PC-8M		55	(2)-PC-12P		80
(1,2)-PC-8P	78		(1,2)-P-16M	62	
(2)-PC-8P		82	(2)-PC-16M		36

[a] Letters a and b refer to the reactions in Figure 2.

(Figures 1 and 2), only one particular example will be given here. Isolated yields for each step and for each lipid are recorded in Tables I and II. Intermediates were invariably characterized by IR, ^{1}H NMR, and ^{13}C NMR. Fatty acids were, in addition, subjected to elemental analyses and precise mass measurements (Table III). Structures of phospholipids were affirmed by IR, NMR, and FAB mass spectral analyses. Complete spectral data are available upon request.[12]

Ethyl Hydrogen Suberate (2).[13,14] Suberic acid (12 g, 0.069 mol) was added to a 1000-mL flask containing 300 mL of ethanol, 360 mL of water, and 3 mL of concentrated sulfuric acid. This mixture was continuously extracted with cyclohexane for 2 days. The aqueous layer was discarded, the cyclohexane cooled, and any unreacted diacid removed by filtration. In order to separate monoester **2** from diester, the cyclohexane was washed with 200 mL (4×) of 1 M aqueous sodium bicarbonate. After acidification with dilute HCl, the monoester was extracted from the aqueous layer with 100 mL (4×) of ether. The ether layer was dried over $MgSO_4$ and stripped leaving 10.2 g (73%) of **2** as a yellow oil.

Ethyl 8-Ketooctadecanoate (3).[15–17] A Grignard solution was prepared under a nitrogen blanket from 1.34 g (0.055 mol) of magnesium turnings, 12.2 g (0.055 mol) of 1-bromodecane, and 100 mL of anhydrous ether. Anhydrous cadmium chloride (5.04 g, 0.028 mol) was added to the stirred solution at 0 °C. After an exothermic reaction had subsided, the ethereal solution of *n*-decylcadmium was heated for 1 h at 40 °C. A Gilman test[18] for Grignard reagent was negative at this point. The ether was then removed by distillation and replaced by 50 mL of dry benzene. Acid chloride (10.1 g, 0.046 mol; prepared by refluxing **2** with thionyl chloride) in 50 mL of dry benzene was added dropwise to the stirred reaction mixture cooled in an ice bath. After the addition was complete, the greenish-yellow solution was boiled under reflux for 1 h with constant stirring. At the end of this period, the flask was cooled in ice and organocadmium complex broken up with 50 g of ice and 50 mL of 6 N sulfuric acid. The benzene layer was then washed successively with 100 mL of water, 5% aqueous sodium bicarbonate, water, and 10% aqueous sodium chloride. The benzene solution was dried and stripped to yield a white semisolid which was crystallized twice from petroleum ether. In order to remove *n*-eicosane (an impurity formed from Grignard coupling), the product was subjected to column chromatography (8:2 petroleum ether/ether, 100–200-mesh Silicar silica gel). Compound **3** was obtained in 47% yield, mp 37 °C (Tables I and III).

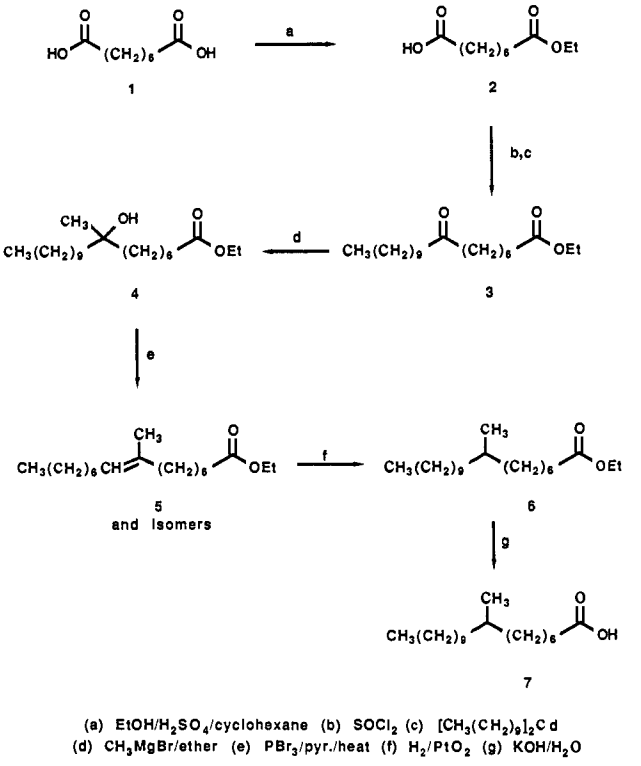

Figure 1. Synthesis of a branched fatty acid.

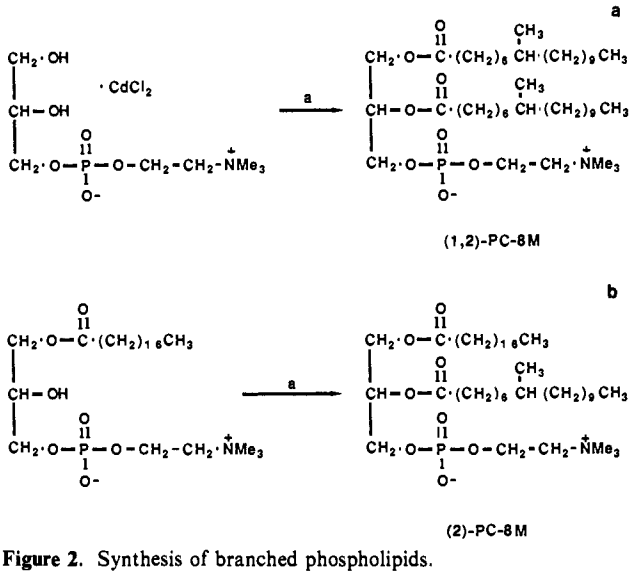

Figure 2. Synthesis of branched phospholipids.

Ethyl 8-Hydroxy-8-methyloctadecanoate (4).[19] Keto ester **3** (6.0 g, 0.018 mol) was added to 30 mL of dry ether in a 200-mL flask (equipped with a mechanical stirrer and reflux condenser) immersed in an ice bath and purged with nitrogen. Following dropwise addition of methylmagnesium bromide (9.2 mL of 2.0 M in ether), the mixture was permitted to stir for 4 h at room temperature. Ice and dilute HCl were added, the ether layer was separated, and the aqueous layer was washed with ether (25 mL, 3×). The portions of ether were combined, washed with 25 mL of water, and filtered through a pad of sodium sulfate. Product **4** was obtained in an acceptable state of purity by stripping the solvent and drying the residue (1 mm, 6 h, 25 °C).

Ethyl 8-Methyloctadec-8-enoate (5).[20] Compound **4** (5.0 g, 0.015 mol) in 2 mL of pyridine and 20 mL of benzene was added to a stirred mixture of phosphorus tribromide (5.4 g) in 2 mL of pyridine. An additional 2 mL of pyridine was added, and the resulting solution was

(12) Spectra and tables of spectral data appear in: Wood, M. G., Jr. Ph.D. Thesis, Emory University, Atlanta, GA, 1988. Detailed mass spectral information have been published: Mattina, M. J. I.; Richardson, S. D.; Wood, M.; Zhou, Q. Z.; Contado, M. J.; Menger, F. M.; Abbey, L. E. *Org. Mass Spectrom.* **1988**, *23*, 292.

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Table III. Melting Points, Elemental Analyses, and Precise Masses of Substituted Octadecanoic Acids

posn	group	mp, °C found	mp, °C lit.	elemtl anal.,[a] % C	elemtl anal.,[a] % H	mass[a,e]	posn	group	mp, °C found	mp, °C lit.	elemtl anal.,[a] % C	elemtl anal.,[a] % H	mass[a,e]
4	butyl	<22		77.55	13.02	340.334 351	12	butyl	<22	23.0–24.5[c]	77.66	13.10	340.332 916
4	methyl	51–52	51.5–52.0[b]	76.56	12.79	298.286 713	12	methyl	35.0–35.5	35.0–35.5[b]	76.54	12.87	298.287 354
8	butyl	<22		77.66	13.07	340.332 825	12	phenyl	<22		79.93	11.19	360.302 216
8	methyl	32.5–33.0	33.0–33.5[b]	76.46	12.87	298.286 362	16	methyl	49–50	49.9–50.6[d]	76.43	12.86	298.287 369
8	phenyl	<22		79.93	11.20	360.303 024							

[a] Butyloctadecanoic acids, mass calcd for $C_{22}H_{44}O_2$, 340.334 130 9. Anal. Calcd: C, 77.58; H, 13.02. Methyloctadecanoic acids, mass calcd for $C_{19}H_{38}O_2$, 298.287 180 7. Anal. Calcd: C, 76.45; H, 12.83. Phenyloctadecanoic acids, mass calcd for $C_{24}H_{40}O_2$, 360.302 830 8. Anal. Calcd: C, 79.94; H, 11.18. [b] Weitzel, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1951**, *287*, 254. [c] Chasin, D. G.; Perkins, E. G. *Chem. Phys. Lipids* **1971**, *6*, 8. [d] Cason, J.; Prout, F. S. *J. Am. Chem. Soc.* **1944**, *66*, 46. [e] Positive ion FAB mass spectra were obtained at an accelerating voltage of 8kV on a VG Analytical 70S mass spectrometer equipped with a VG 11/250 data system. Triethanolamine served as a matrix, and CsI as the mass calibrant. Masses are accurate to only six significant figures.

refluxed for 4 h with the aid of an oil bath set at 90 °C. The reaction mixture was cooled to 0 °C, followed by addition of 50 mL of water and by an ether extraction (50 mL, 3×). The extract was washed first with dilute sulfuric acid (to eliminate the pyridine) and then with aqueous sodium bicarbonate. Finally, the ether was dried and stripped to produce a yellow oil which was purified by chromatography (9:1 petroleum ether/ether; silica gel). Compound **5**, a mixture of positional isomers, was obtained as a clear oil with satisfactory spectral properties.

Ethyl 8-Methyloctadecanoate (6). Compound **5** (3.7 g 0.011 mol), platinum(IV) oxide (0.37 g), and 50 mL of absolute ethanol were added to a Parr bottle which was then placed on a Parr shaker apparatus. Olefinic reduction at 50 psi was allowed to proceed for 2 days. The workup consisted of degassing with N_2, filtering through Celite, and stripping the ethanol under reduced pressure to given an orange oil. Chromatography, under conditions identical with those described with compound **5**, gave a colorless oil which was characterized in the usual manner.

8-Methyloctadecanoic Acid (7). The ethyl ester **6** (3.0 g, 9.2 mmol) was added to a round-bottomed flask along with 95% ethanol (30 mL) and 2.5 N aqueous sodium hydroxide (30 mL). Magnetic stirring and refluxing were carried out for 4.5 h, after which the ethanol was removed to give a thin gel. This gel was mixed with 200 mL of water, acidified, and extracted with ether (150 mL, 3×). Removal of the dried ether gave 2.5 g (91%) of **7**, a white acidic solid. This material was crystallized five times from HPLC-grade acetone to give product with the correct melting point, elemental analysis, and spectra (Table III). Great care was always taken to purify the final fatty acids because, as explained in the next section, high purity is critical for film balance work.

1,2-Bis(8-methylstearoyl)-*sn*-glycero-3-phosphatidylcholine (8).[21-23] L-α-Glycerophosphorylcholine/$CdCl_2$ complex (89.6 mg, 0.196 mmol; purchased from Avanti and dried for 24 h at 56 °C and 0.1 mmHg) was mixed with fatty acid **7** (239 mg, 0.80 mmol) and 4-(dimethylamino)-pyridine (48 mg, 0.40 mmol) in 2 mL of chloroform (freshly distilled from P_2O_5). Dichlorohexylcarbodiimide (165 mg, 0.80 mmol) was added, whereupon the mixture was stirred for 7 days at room temperature in the dark under a blanket of nitrogen. The entire reaction mixture including the solids was placed on a column (5.5 g of Silicar silica gel in a 1 × 20 cm column) and the product isolated by chromatography. Eluting solvents consisted of various mixtures of chloroform and methanol (20-mL fractions):

vol, mL	$CHCl_3$, %	CH_3OH, %
100	100	0
200	90	10
100	80	20
100	50	50
200	20	80

The lipid fraction was purified again, this time with the following regime:

vol, mL	$CHCl_3$, %	CH_3OH, %
100	100	0
100	90	10
100	80	20
200	50	50
200	20	80

Phospholipid **8** was obtained in 81% yield. Its purity was judged satisfactory by TLC analysis (a single sharp spot on silica plates eluted with 65:25:4 $CHCl_3/CH_3OH/H_2O$ and developed with I_2 followed by Dragendorff's reagent),[24] NMR, and FAB mass spectrometry.[12]

1-Stearoyl-2-(8-methylstearoyl)-*sn*-glycero-3-phosphatidylcholine (9). Monostearoyllecithin (236 mg, 0.45 mmol; purchased from Avanti and dried for 24 h at 25 °C and 1 mmHg) and 8-methylstearoyl anhydride (579 mg, 1.0 mmol; synthesized via a known procedue)[23] were dissolved in 15 mL of chloforom (freshly distilled from P_2O_5). 4-(Dimethylamino)pyridine (110 mg, 0.9 mmol) was then added, whereupon the mixture was magnetically stirred under nitrogen for 12 h in the dark. The solvent was stripped, and the residue, suspended in ~1 mL of chloroform, was chromatographed twice (as described directly above) to give 200 mg (55%) of phospholipid.

Film Balance Operation. Pressure vs area curves were obtained with the aid of Fromherz film balance (Mayer Feintechnik, Postfach 2864 3400 Gottingen, West Germany) equipped with a Teflon multicompartment trough and Wilhelmy plate device.[25] The instrument rested upon a vibration-free table (Micro-g, Technical Manufacturing Corp., Peabody, MA). The tubular drive assembly of the film balance was dropped through a 20-cm hole bored into the table. A Lauda circulating bath provided thermostating for the balance. A Fisher x-y recorder allowed continuous monitoring of the pressure–area isotherms.

Formidable experimental difficulties are associated with film balance research. In this connection Fendler wrote, "All published work should...be scrutinized for experimental details. Those that do not provide adequate descriptions...should be viewed with suspicion."[26] Since we agree with this sentiment, an extensive account of our procedures will be provided.

Equipment and glassware were purchased new and dedicated to the film balance work. The glassware was initially cleaned with Nochromix/H_2SO_4 and rinsed with purified water. Thereafter, glassware was cleaned by rinsing with HPLC hexane, soaking in 20% ether/ethanol, and rinsing with purified water. Glassware was stored at 60 °C in a clean oven until needed. Aluminum foil, used to cover beakers and working surfaces, was treated in an identical manner. The film balance trough was cleaned initially with Nochromix/H_2SO_4 and rinsed repeatedly with purified water. Prior to initial runs, water was allowed to remain in the trough for many weeks (during which it was changed daily) in order to leach out possible contaminants in the Teflon. From time to time between experiments, the trough was wiped with high-quality filter paper (not Kimwipes!) soaked in 20% ether/ethanol, a good solvent for our lipids.

The efficiency of our table isolator was tested by comparing pressure–area curves while the water purification system, constant-temperature bath, movement of personnel, and other potential sources of vibration were and were not in progress. No difference was observed.

Reasonable care was taken to keep the film balance dust-free. A separate room, free of any synthetic work, was used to house the balance and accompanying equipment. The room had sealed windows, filters over the heating vent, and shed-free (plastic-coated) ceiling tiles. A dust precipitator (Sears) operated continually. The floor was occasionally subjected to a vacuum cleaner dedicated to the room. Personnel always removed their shoes prior to entry into the room and replaced them with slippers. These precautions were taken despite our observation that dust particles, purposely placed above and below the film, had only small

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effects on the pressure–area curves.

Purity of lipid, aqueous subphase, and solvents is obviously critical (only 10^{-8} mol of material is needed to create a film). Thus, solid fatty acids were crystallized at least five times from HPLC acetone, whereas liquid fatty acids were chromatographed three times (retaining center cuts only). Phospholipids were chromatographed two or three times until TLC and spectral analyses[12] gave no detectable impurities. Water, prepurified by means of three large tanks containing deionizers and charcoal, was directed into a four-cartridge Millipore Milli-Q system including a terminal Organix-Q cartridge for removing trace organics and a 0.22-μm membrane filter for removing microscopic organisms and particles. Water was maintained at a resistivity of 18 megaohm·cm and a surface tension of 72.4 ± 0.3 dyn/cm (25.0 °C; Fisher Tensiomat). All organic solvents (except ether) were HPLC-grade. Work was carried out while wearing disposable polyethylene gloves (Fisher) to avoid possible contamination by skin lipids.

A platinum–iridium wire, used for pressure calibration of the balance, was made according to Fromherz specifications (326.40 mg = 100 dyn/cm for a 15.0 × 11.5 × 0.1 mm Wilhelmy plate). The plates were cut from low-ash filter paper with the aid of a template and razor knife. As an extra precaution, a fresh paper plate was used for each pressure–area curve; this required calibration prior to every run. Plates were hung on the film balance hook by means of a tiny hole punched 2 mm below the 11.5-mm edge (the template providing a reproducible location of the hole). Since pressure–area curves showed slight variations when the plate was deliberately tilted from perpendicular (off-radial rotation was less important), care was taken to position the plate vertically.

Monolayers were spread from 10^{-4} M fatty acid (or fatty acid mixtures) in hexane or phospholipid in chloroform. From 40 to 100 μL were delivered in at least 10 locations across the water surface with a Hamilton microliter syringe equipped with a Chaney adapter. Typically, the initial enclosed surface area encompassed 90 cm^2. At least 20 min was allowed for the spreading solvent to evaporate and for the lipid molecules to distribute themselves evenly throughout the film. The subphase consisted of 0.01 M H_2SO_4 in purified water for the fatty acids, or purified water for the phospholipids.

Pressure–area isotherms were recorded at 23.0 ± 0.1 °C and at barrier speeds of ~4 Å^2/molecule per min. Increasing or decreasing the barrier speed by 2 Å^2/molecule per min had no effect on the isotherms except for a slight variation in the pressure at which the film collapses. Alongside the film balance, and within its plastic housing, open beakers of water with cloth wicks kept the relative humidity at 70–85% (Airguide hygrometer). A high relative humidity is desirable to minimize evaporative loss of water from the trough.[27]

The quality of the aqueous subphase was tested prior to each run by compressing the surface without a lipid film while noting any rise in surface pressure. If an increase was detected, the subphase was removed by aspiration through disposable pipets (previously washed with Nochromix/H_2SO_4), and the trough was cleaned again with 20% ether/ethanol. A study of pressure–area curves vs height of subphase indicated that reproducible results could be obtained if the subphase level was kept 1–2 mm above the edge of the trough. Under such conditions, no leakage of film occurred under the movable barrier (as judged by lack of movement in a talc layer spread outside the enclosed area).

Before beginning film balance work with our lipids, we checked our technique against two trustworthy pressure–area isotherms in the literature: pentadecanoic acid carried out by Pallas and Pethica[27] and stearic acid carried out by Feher, Collins, and Healy.[28] Periodically throughout the course of our work, we returned to these standards to ensure that some miscreant had not unknowingly surfaced. It was often possible to detect impurities in a lipid by a "hump" in the pressure–area curve. Thus, Aldrich stearic acid (>99%) and Avanti L-α-diastearoylphosphatidylcholine (>99%) but not Fluka stearic acid (>99.5%) manifested an impurity. Our own lipids gave "hump-free" curves with run-to-run area reproducibility of 2% at 10 dyn/cm. All pressure–area curves were carried out in duplicate.

Results and Discussion

Pressure–area curves were secured from the following system: (a) stearic acid substituted at four locations (4, 8, 12, and 16) with methyl groups, at three locations (4, 8, and 12) with *n*-butyl groups, and at two locations (8, 12) with phenyl groups; (b) mixtures of two fatty acids in various proportions (e.g., 75% 8-methyl + 25% 16-methyl); (c) phospholipids where either both chains had substituents at varying positions or only the second chain was derivatized. (Film balance experiments were also carried out on lipids with polar groups, but these will be reported in a future publication.) Although our amassing this huge amount of data was desirable from the standpoint of detecting subtleties in packing behavior, it is obviously not feasible to present here our entire library of pressure–area curves.[29] Instead, we will provide the reader with sufficiently many representative plots so that the important generalizations arising from the study can be understood and believed.

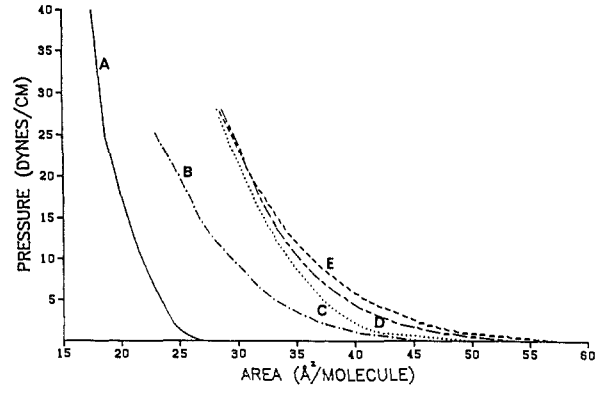

Figure 3. Pressure–area isotherms (23.0 °C) for (A) stearic acid, (B) 16-methylstearic acid, (C) 12-methylstearic acid, (D) 4-methylstearic acid, and (E) 8-methylstearic acid.

Pressure–area isotherms at 23 °C for stearic acid and four methylstearic acid isomers are shown in Figure 3. These plots were obtained by compressing monolayers of fatty acids resting on a 0.01 M H_2SO_4 subphase (see Experimental Section for full details). Two points are readily apparent from Figure 1: (a) The area-per-molecule is greater for the four methylstearic acids than for stearic acid itself. In other words, methyl substitution "expands" the film (a fact already well established in the literature[6,7]). (b) Packing areas of methyl-branched stearic acids follow the sequence of $16 < 12 < 4 < 8$. Thus, at 5 dyn/cm pressure, the 16-, 12-, 4-, and 8-methylstearic acids occupy 33, 37, 39, and 41 Å^2/molecule, respectively, compared to 23 Å^2/molecule for the parent acid.

Immediately, we encounter a problem that, unfortunately, has not yet been fully resolved in the literature: How does one extract, for theoretical purposes or otherwise, molecular areas from pressure–area curves? It is possible, of course, to select an arbitrary film pressure (e.g., 5 dyn/cm as in the preceding paragraph) and record areas at that particular pressure. Although this popular approach suffices for qualitative comparisons, the resulting areas are pressure-dependent. To obtain "intrinsic" areas, a more laborious analysis, such as that recently exploited by the Kaiser group,[30] is required.

The method is based on a two-dimensional variation of a nonideal gas law (eq 1) where π = the observed pressure (dyn/cm), A = the observed film area (cm^2), A_∞ = the "limiting area" (cm^2),

$$\pi(A - A_\infty[1 - k\pi]) = nRT \qquad (1)$$

an *empirical* parameter approximating the area occupied by the molecules at zero pressure (which, for convenience, is then converted to Å^2/molecule), k = the compressibility constant (found to equal (1–2) × 10^{-2} cm/dyn for our systems), n = the apparent number of moles lipid in the film, R = 8.31 dyn·cm/mol·K, and T = K. Equation 1 does not apply to the entire pressure–area curve; it is restricted to the "liquid" phase of the film. In order to define the region obedient to eq 1, we plotted $\pi_2A_2 - \pi_1A_1$ vs π_1 where, typically, $\pi_2 - \pi_1 = 1$ dyn/cm. It can be shown that whenever this plot is linear (e.g., the 3–10 dyn/cm region for our fatty acids), eq 1 is applicable. Parameters A_∞,k, and n were then

(27) Pallas, N. R.; Pethica, B. A. *Langmuir 1985, 1*, 509. An l_e/l_c transition pressure of 7.1 dyn/cm was in close agreement with the Pallas and Pethica value.

(28) Feher, A. I.; Collins, F. D.; Healy, T. W. *Aust. J. Chem.* **1977**, *30*, 511.

(29) Data are available upon request.

(30) Taylor, J. W. Ph.D. Thesis, University of Chicago, 1983.

Table IV. Limiting Areas A_∞, Collapse Areas A_c, and Calculated Areas A_{calcd} for Substituted Fatty Acids[a]

compd[b]	A_∞	A_c	A_{calcd}	compd[b]	A_∞	A_c	A_{calcd}
SA	21		24	4-BSA	51	38	51
4-MSA	39	29	34	8-BSA	51	40	54
8-MSA	41	29	37	12-BSA	42	34	54
12-MSA	38	28	37	8-PSA	54	41	59
16-MSA	33	23	31	12-PSA	45	34	59

[a] All areas, Å²/molecule. [b] SA, stearic acid; M, methyl; B, *n*-butyl; P, phenyl.

calculated by fitting the pressure–area data in the "liquid" region to eq 2 (a rearranged form of eq 1) by a trivariant linear regression.

$$\pi A = nRT + \pi A_\infty - \pi^2 kA_\infty \quad (2)$$

A_∞ values for the racemic[31] methyl-, *n*-butyl-, and phenyl-substituted stearic acids are listed in Table IV.

A_∞ values characterize molecular areas in the so-called "liquid-expanded" region of the films. The curved portions of the plots in Figure 1 (below ca. 10 dyn/cm) encompass this region. Above 10 dyn/cm, the plots become more linear owing, very likely, for formation of "solid" domains (i.e., islands of close-packed lipid).[32] The presence of two phases, liquid and solid, may account for the deviations of our pressure–area data from eq 1 above 10 dyn/cm. Typically, a "liquid-expanded" region has a molecular area twice that of the molecular cross-section (20 Å² for a hydrocarbon chain[3]). Since liquid hydrocarbons are only slightly less dense than the corresponding solid, the average intermolecular distance is far greater in a "liquid" film than in a bulk liquid. Liquid and "liquid" films are, therefore, not analogous. A "liquid-expanded" film is best viewed as an array of chains that protrude from the water surface and that lie, at least partially, in van der Waals contact with each other. However, the chains are not, on the average, positioned vertically as they are in close-packed "solid" films formed under higher pressures.

Table IV shows that the "liquid-expanded" region is sensitive to the size and location of the chain substituent: (a) As already mentioned, 16-methylstearic acid has by far the lowest A_∞ among the four isomers. Thus, its A_∞ = 33 Å²/molecule falls well below the 38–41 Å²/molecule range for the 4-, 8-, and 12-compounds. (b) Not surprisingly, an *n*-butyl group expands the film more effectively than does a methyl substituent at the same location (e.g., 51 and 39 Å²/molecule for the 4-position, respectively). The 12-*n*-butylstearic acid has an area almost 9 Å²/molecule less than that of the 4- and 8-butyl fatty acids. (c) Phenylstearic acids pack similarly to the corresponding *n*-butyl compounds. Again, the 12-isomer occupies a 9 Å²/molecule smaller area than the 8-isomer.

Micelles do not display the sensitivity to substituent location seen with the films. Thus, the critical micelle concentrations for the 4-, 8-, 12-, and 16-methylstearic acids (determined tensiometrically in 0.01 N KOH) equal 4.37, 4.87, 5.03, and 4.85 × 10^{-5} M, respectively. Lack of discrimination among the isomers is evidence that micelles are highly disorganized assemblages.

When a film is compressed by means of a movable barrier, the pressure ultimately becomes too high for the film to maintain its integrity. The film collapses as the molecules "pile up". Experimentally this is observed by an abrupt drop in the pressure–area curve. Collapse pressures for the alkyl- and aryl-substituted stearic acids vary from 20 to 29 dyn/cm compared to >50 dyn/cm for stearic acid. Thus, substitution reduces (as expected[3]) film stability. Collapse areas A_c, listed in Table IV, are seen to parallel the trends described for A_∞. Only peripheral mention will be made of collapse parameters owing to the danger that highly compressed films lie far from equilibrium even with slow compression rates.[33]

In an effort to understand packing interactions on a molecular level, we have examined the space-filling properties of our branched

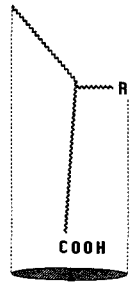

Figure 4. Schematic used in SHADOW calculations to obtain areas of occupation, A_{calcd}, listed in Table IV.

Table V. Limiting Areas A_∞, Ideal Mixing Areas A_{av}, and Collapse Pressures P_c for Mixed Monomolecular Films[a]

components[b]				
%	compd	A_∞	A_{av}	P_c
100	SA	21	21	>50
5:95	8-MSA/SA	24	22	29
10:90	8-MSA/SA	24	23	30
25:75	8-MSA/SA	29	26	27
50:50	8-MSA/SA	35	31	29
75:25	8-MSA/SA	38	36	26
100	8-MSA	41	41	28
100	SA	21	21	>50
25:75	8-BSA/SA	30	29	22
50:50	8-BSA/SA	40	36	22
75:25	8-BSA/SA	48	44	26
100	8-BSA	51	51	22
100	16-MSA	33	33	25
25:75	8-MSA/16-MSA	38	35	29
50:50	8-MSA/16-MSA	39	37	31
75:25	8-MSA/16-MSA	40	39	29
100	8-MSA	41	41	28

[a] All areas, Å²/molecule; pressure, dyn/cm. [b] SA, stearic acid; M, methyl; B, *n*-butyl.

fatty acids via a combination of force field and analytical programs given the name SHADOW.[34] Information provided by these computations was then compared with that obtained experimentally with the film balance. Although approximate, the calculations, based on static ground-state geometrics, simulate reality surprisingly well.

SHADOW software utilizes MACROMODEL[34] to generate minimum energy conformations of the substituted fatty acids. Torsional and bond angle displacements at points of substitution induce chain bending evident in Figure 4. (Gauche linkages at nonsubstituted sites were not incorporated into the structures.) Next, the program pivots the energy-minimized fatty acid about its carboxyl proton lying in the horizontal plane until the smallest "shadow" projected onto this plane is located. The areas occupied by the shadows, recorded as A_{calcd} in Table IV, can be easily reckoned from van der Waals radii of the atoms comprising the fatty acids.

Evidence for the validity of A_{calcd} comes from early film balance studies of Weitzel et al.[7] These workers found that the "kondensation-punkt" (i.e., the area where the pressure–area curve leaves the base line) is higher at constant sensitivity for substitution at the center of the chain than at the extreme ends. Our calculations bear this out. Thus, the SHADOW model predicts that as the site of methyl substitution shifts from 2 to 10 to 17, the area will vary from 31 to 37 to 30 Å²/molecule for stearic acid. A distortion at the center most effectively expands the area required by an otherwise linear chain.

Agreement between A_{calcd} and A_∞ values in Table IV is good except for the 12-*n*-butyl and 12-phenyl fatty acids, where abnormally low molecular areas were observed on the film balance. Our inability to predict these low areas undoubtedly reflects the static nature of the SHADOW model. A variety of data has shown

(31) For a comparison of optically active and racemic 12-hydroxystearic acid, see: Tachibana, T.; Hori, K. *J. Colloid Interface Sci.* **1977**, *61*, 398.
(32) Bell, G. M.; Combs, L. L.; Dunne, L. J. *Chem. Rev.* **1981**, *81*, 15.
(33) Reference 3, p 144.
(34) We thank Professor Clark Still for this program. A Fortran 77 listing of the SHADOW program is available upon request.

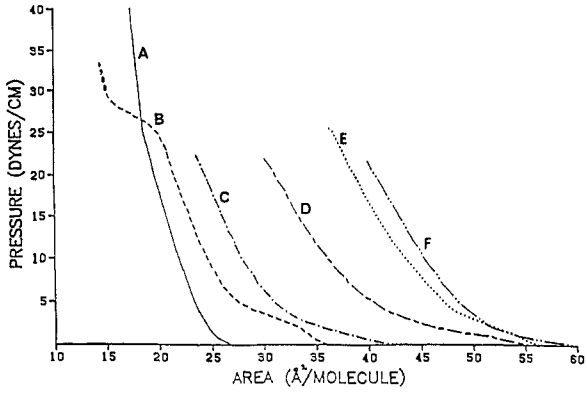

Figure 5. Pressure–area isotherms (23.0 °C) for (A) stearic acid (SA), (B) 10% 8-butylstearic acid (8-BSA) + SA, (C) 25% 8-BSA + SA, (D) 50% 8-BSA + SA, (E) 75% 8-BSA + SA, and (F) 8-BSA. See curves except A terminate at the collapse of the film.

that conformational freedom in bilayers (composed of two stacked monolayers) increased abruptly beyond the center of the chains.[35,36] Carbon segments near the chain termini have, consequently, greater room to fold and to thereby minimize the perturbations caused by large pendant groups. We are currently developing the molecular dynamics software necessary to treat such behavior.[37]

It is a natural extension of the work just described to inquire how *mixtures* of our fatty acids pack in monomolecular films. Data relevant to this question are found in Figure 3 and Table V. But before contemplating the significance of these results, we must first introduce the subject of mixed films with a few remarks. A mixed monolayer can be categorized into three types: complete miscibility, total phase separation, and partial miscibility (Figure 6, parts a–c, respectively). Both "complete miscibility" and "total phase separation" obey eq 3 where the observed mo-

$$A_{av} = N_1A_1 + N_2A_2 \qquad (3)$$

lecular area of the mixture, A_{av}, is a weighted average of the individual areas (the Ns being mole fractions of the components). "Miscible" (or "homogeneous") films conform to eq 3 because the components do not discriminate among each other and, therefore, they distribute randomly; the total area is a simple summation of the individual areas. An "immiscible" film, characterized by large patches of A_1 coexisting with large patches of A_2, also exhibits ideal mixing behavior provided that boundary molecules are relatively few in number (e.g., <1% with patches of 10^5 members[3]). Deviations from eq 3 are observed whenever special interactions among miscible molecules perturb the additivity of the areas. Since this happens to be the situation with our fatty acids, we will address nonideality more specifically in the next paragraph.

Figure 5 displays pressure–area curves for stearic acid (A_∞ = 21 Å²/molecule) mixed with 8-butylstearic acid (51 Å²/molecule). A_∞ values for three different pairs of fatty acids, along with A_{av} values calculated assuming obediance to eq 3, are listed in Table V. It is seen that the observed areas, A_∞, invariably exceed ideality. In order to understand the positive deviations from eq 3, we have constructed a highly schematic and simplified model (Figure 7). Assume that stearic acid (SA) and 8-*n*-butylstearic acid (8-BSA) occupy 1 and 2 area units per molecule, respectively. Assume also that two adjoining 8-BSA molecules occupy an area of 3, rather than 4, because a rough film surface permits interdigitation as pictured in Figure 7. Pure "four-molecule" films of SA and 8-BSA require, therefore, 4 and 6 area units, respectively. The area covered by a "two + two" mixed film of SA and 8-BSA depends upon the particular arrangement of the four molecules. If the two 8-BSA molecules are in contact, the total area should equal 5, a number identical with that calculated from eq 3 (i.e.,

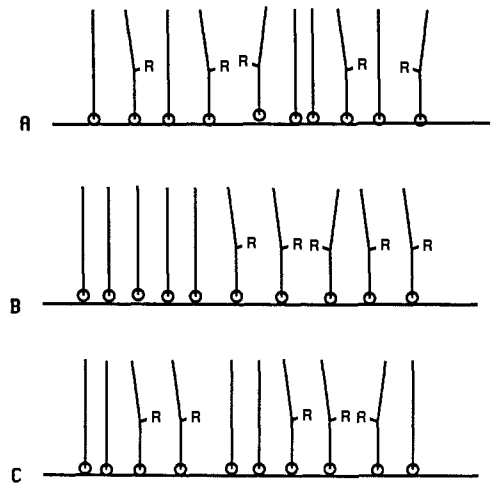

Figure 6. Schematic diagrams of three different types of mixed films: (A) completely miscible (homogeneous); (B) immiscible (phase separation); (C) partially miscible. Based on Figure 6.1 in ref 3.

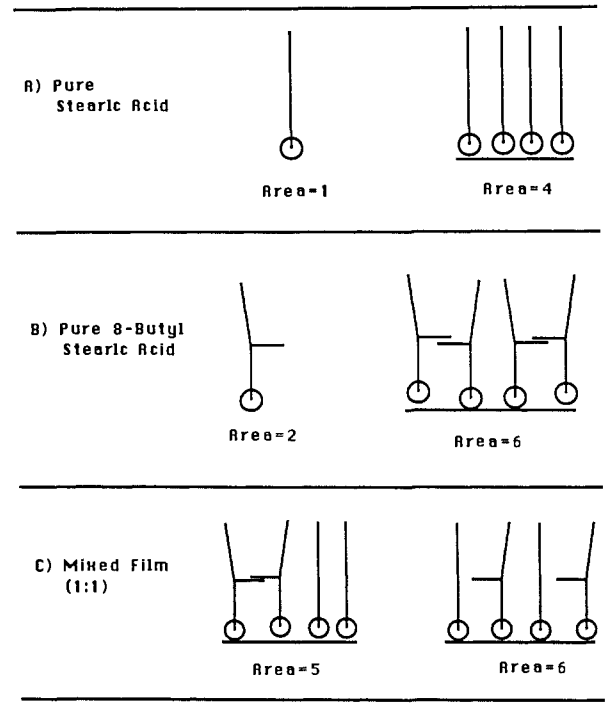

Figure 7. Schematic showing how interdigitation can explain molecular areas in mixed films greater than ideality (see text).

0.50 × 4 + 0.50 × 6). If, on the other hand, the 8-BSA molecules are separated from each other by intervening SA molecules, then the total area equals 6 units. Therefore, to the extent that 8-BSA molecules are isolated within a film, the A_∞ value will exceed ideality as was, in fact, observed. Although our model may not be the only explanation for positive deviations from eq 3, it is certainly reasonable to postulate vertical slippage of the chains to reduce steric interactions. When this happens, area additivity no longer prevails.

One additional point about the mixed films pertains to their collapse pressures given in Table V. Stearic acid forms a stable condensed film that collapses only when the pressure reaches ~50 dyn/cm.[38] All our substituted stearic acids collapse at considerably lower pressures (e.g., 22 dyn/cm for 8-BSA). Low collapse pressures are also observed when even small amounts of branched fatty acid are admixed with stearic acid (e.g., 29 dyn/cm for 5% 8-MSA in SA). Figure 5, in which the presure–area curves terminate at the collapse pressures, illustrates the effect. Clearly,

(35) Seelig, A.; Seelig, J. *Biochemistry* **1974**, *13*, 4839.
(36) Williams, E.; Sears, B.; Allerhand, A.; Cordes, E. H. *J. Am. Chem. Soc.* **1973**, *95*, 4871.
(37) Northrup, S. H.; Curvin, M. S. *J. Phys. Chem.* **1985**, *89*, 4707.

(38) Collapse pressures are approximate owing to the steep curves and to their dependence on experimental conditions (e.g., sweep rate).

Table VI. Limiting Areas A_∞, Collapse Areas A_c, and Transition Temperatures T_c for Substituted Phosphatidylcholines[a]

lipid[b]	A_∞	A_c	T_c	lipid[b]	A_∞	A_c	T_c
DSPC	56	38	55	(2)-PC-12M	89	46	8
(1,2)-PC-4M	86	44	30	(2)-PC-16M	57	43	39
(1,2)-PC-8M	98	49	<3	(1,2)-PC-8P	98	65	<3
(1,2)-PC-12M	90	56	<3	(1,2)-PC-12P	94	65	<3
(1,2)-PC-16M	84	49	27	(2)-PC-8P	96	61	<3
(2)-PC-4M	82	43	41	(2)-PC-12P	91	57	<3
(2)-PC-8M	90	41	19				

[a] All areas, Å^2/molecule; temperatures, °C. [b] See introduction for structure notation.

an excess of stearic acid cannot prevent domains of branched fatty acid from forming and buckling under stress. As a result, branched fatty acid is selectively extruded from the mixed film. The pressure–area curve for 10% 8-BSA/90% SA in Figure 5B is particularly revealing: Formation of "bulk" 8-BSA (containing, perhaps, small amounts of SA) can be detected at 25 dyn/cm where the plot levels off. Above 30 dyn/cm, the SA film, purified of its 8-BSA, continues to compress more or less normally.

Finally, we will describe briefly experiments with phospholipids bearing substituted fatty acid chains.[39] Table VI lists 12 such phospholipids, their A_∞ values, and their collapse areas A_c. Also listed in Table VI are the phase-transition temperatures T_c (determined by NMR[40] and by differential scanning calorimetry[41,42] on bilayer systems). Note that the pressure–area isotherms were all secured at 23.0 °C, a temperature that lies both below and above the various T_c values in Table VI. This should not complicate the comparisons of areas, however, because A_∞ reflects the "liquid-expanded" region for all our compounds. In order to obtain a close-packed arrangement, one can either compress a "liquid-expanded" film or else cool a bilayer below its T_c. Temperature variation studies, now in progress, will provide more information on monolayer–bilayer relationships.

Several generalizations are evident from Table VI: (a) The area expands from 56 Å^2/molecule for distearoylphosphatidylcholine (DSPC) to 84–98 Å^2/molecule for methyl substitution in *both* chains at the 4-, 8-, 12-, and 16-positions. (b) Methyls, placed exclusively on the second chain, also expand the areas into the 80 range except for the 16-isomer where the methyl has a negligible effect. A relatively small perturbation by a 16-methyl was also noted with simple fatty acids (Table IV). Comparison of lipid substituted at both 16-positions (A_∞ = 84 Å^2/molecule) with the corresponding singly substituted material (A_∞ = 57 Å^2/molecule) shows that one, but not two, distal methyls per lipid can be tolerated by a "liquid-expanded" film without notable expansion. (c) T_c data indicate that the most effective bilayer "melting" (i.e., the lowest gel-to-liquid crystal transition temperature) occurs when the methyl groups reside near the center of the chain. Thus, T_c = 41, 13, and 39 °C for lipid that is singly substituted at the 4-, 12-, and 16-positions, respectively. (d) Phenyl groups at positions 8 and 12, although lowering T_c dramatically, perturb the "liquid-expanded" film only slightly more than do methyl groups (e.g., A_∞ = 94 and 90 Å^2/molecule for (1,2)-PC-12P and (1,2)-PC-12M, respectively). This is not true for close-packed films near the collapse pressure where a phenyl is far more disruptive than a methyl. Thus, A_c increases 9 Å^2/molecule when a methyl at carbon 12 is replaced by a phenyl. The magnitude of an observed steric effect is, of course, dictated by the state of the film.

In summary, we have synthesized a series of highly purified fatty acids and phospholipids possessing chain substituents of varying size (methyl, *n*-butyl, and phenyl) and at varying locations (carbons 4, 8, 12, or 16 of an 18-carbon chain). Pressure–area isotherms provided information on how these molecules, either individually or admixed, pack in monomolecular films. Preliminary calculations could, in many cases, provide film areas in reasonable agreement with experiment, but more sophisticated theoretical work, currently underway, is needed for a complete understanding at the molecular level. We are also examining, now that the packing behavior has been systematically investigated, how chain substitution influences transport rates through membranes constructed of the various lipids.

Acknowledgment. This work was supported by the National Institutes of Health and the Army Research Office. We thank Jud Virden (3M Corp.) for advice in handling the film balance and Dr. M. J. I. Mattina for the FAB mass spectra.

(39) For film studies on diisoeicosanoylphosphatidylcholine, see: Suzuki, A.; Cadenhead, D. A. *Chem. Phys. Lipids* **1985**, *37*, 69.

(40) Feigenson, G. W.; Chan, S. I. *J. Am. Chem. Soc.* **1974**, *96*, 1312. The NMR method described therein was used to estimate T_c values. The values agreed well with those obtained later by DSC although the latter are far more accurate.

(41) We thank Professor Harry Hopkins, Georgia State University, for assistance with the DSC measurements. A detailed collaborative report on thermotropic properties can be found in the following paper in this issue.

(42) For discussions of thermotropic properties of various branched-chain lecithins, see: (a) Silvius, J. R.; Lyons, M.; Yeagle, P. L.; O'Leary, T. J. *Biochemistry* **1985**, *24*, 5388. (b) Nuhn, P.; Brezesinski, G.; Dobner, B.; Förster, G.; Gutheil, M.; Dörfler, H. D. *Chem. Phys. Lipids* **1986**, *39*, 221. (c) Lewis, R. N. A. H.; Sykes, B. D.; McElhaney, R. N. *Biochemistry* **1987**, *26*, 4036.