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Monolayer Studies with Synthetic Saturated, Mono- and Polyunsaturated Mixed 1,2-Diglycerides, 1,2-Diacylphosphatidylethanolamines and Phosphatidylcholines at the Air-Water-Interface

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Summary: In this paper we describe physico-chemical properties of monomolecular films of nine synthetic phosphatidylethanolamines (cephalins), six phosphatidylcholines (lecithins) and five 1,2-diglycerides. These compounds carry in the 1-position a saturated fatty acid (stearic acid) and in the 2-position stearic-, oleic-, linoleic-, α-linolenic-, arachidonic- and phytanic acid as acyl groups. The state of the film and its temperature dependence is determined by the chemical structure (number of *cis*-double bonds) of the hydrocarbon chain of the fatty acids. Lipids containing polyenoic fatty acids form only monolayers in the liquid expanded state in the temperature range between 10°C and 40°C, which we investigated. Also the influence of the different hydrophobic and hydrophilic groups on the compressibility, the energy of compression, the pressure and molecular area at the collapse point has been described. The dependence of these parameters on the temperature has been studied.

Zusammenfassung: *Untersuchungen an monomolekularen Filmen verschiedener synthetischer einfach- und mehrfach ungesättigter 1.2-Diglyceride, 1.2-Diacyl-phosphatidyläthanolamine und Phosphatidylcholine an der Luft-Wasser-Grenzfläche.* In der vorliegenden Arbeit werden physikalisch-chemische Eigenschaften monomolekularer Filme von 9 synthetischen Phosphatidyläthanolaminen (Kephalinen), 6 Phosphatidylcholinen (Lecithinen) und 5 1.2-Diglyceriden, die in der 1-Stellung mit einer gesättigten (Stearinsäure) und in der 2-Stellung mit Stearin-, Öl-, Linol-, α-Linolen-, Arachidon- oder Phytansäure acyliert sind, beschrieben. Der Filmzustand und seine Temperaturabhängigkeit wird durch die chemische Struktur (Anzahl der *cis*-Doppelbindungen) der Kohlenwasserstoffketten der Fettsäuren bestimmt. Polyensäurehaltige Lipoide bilden im untersuchten Temperaturbereich von 10°C bis 40°C einen flüssig expandierten Film. Ferner werden der Einfluß der verschiedenen hydrophoben und hydrophilen Gruppen auf die Kompressibilität, die Kompressionsenergie, den Kollapsdruck und den molekularen Flächenbedarf am Kollapspunkt und die Abhängigkeit dieser Parameter von der Temperatur beschrieben.

Complex lipids represent essential components of biological membranes. Particularly phosphatidylcholines and phosphatidylethanolamines are the two main classes present in the lipidphase of the membranes. Therefore their physico-chemical properties should have a significant influence on the properties of membranes. Whatever the molecular fine structures of membranes are, the properties of the components always determine the parameters with regard to the structure and the reactivity of the membranes.

For the study of physico-chemical properties of

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Enzyme:

Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3).

lipid molecules and particularly of the amphipathic phospholipid molecules, the lipid monolayer represents a simple model. LANGMUIR's technique[1] has been used in many earlier studies first with fatty acids, then with naturally occurring phospholipids. LEATHES[2,3] studied the properties of monolayers of egg lecithin, hydrogenated lecithin and cholesterol. He described the expanded liquid state of natural lecithins and the condensed state of hydrogenated lecithin and cholesterol films. ADAM[4,5], DERVICHIAN[6–8], DE BERNARD[9,10] and WATKINS[11] investigated mixed films of phospholipids with fatty acids, glycerides and cholesterol. Valuable contributions to our understanding of the binding forces in natural membranes were made by ELEY and HEDGE[12,13], SCHULMAN[14,15] and PAYENS[16], who studied interactions of protein and phospholipid monolayers. Recently monolayer studies were carried out with synthetic saturated and monoenoic phospholipids[17–19]. The limitation of these investigations are given by the fact, that only saturated phospholipids or mixtures of phospholipids with ill defined distribution of their acylgroups have been used. Naturally occurring membranes however contain predominantly phosphatidylcholins and -ethanolamines, which are substituted in the 1-position with saturated fatty acids, but in the 2-position with monoenoic-, dienoic-, trienoic- and tetraenoic fatty acids such as oleic-, linoleic-, linolenic- and arachidonic acid.

VAN DEENEN and his coworkers[20,21] determined the force-area (F/A) isotherms of a number of synthetic mixed fatty acid phosphatides by means of a conventional LANGMUIR balance. Their force measurement reached to about 50 dyn/cm, thus not reaching the collapse point. Areas of between 35 and 200 Å were measured. The F/A measurements of CHAPMAN[17] on DL-lecithins and cephalins with stearate, oleate and elaidate were made in the same range. CHAPMAN *et al.*[17] and SHAH and SCHULMAN[15] observed that cholesterol in mixed films with phospholipids exerted a condensing effect. In these monolayer studies the monomolecular films were regarded as static structures. More important for the stability of phospholipid films however appear to be their stability, their energetic changes and dynamic properties.

In this publication we wish to report upon monolayer studies with synthetic diglycerides, phosphatidylcholines and phosphatidylethanolamines, which were substituted in position 1 of the glycerol-3-phosphate backbone with stearic acid and in position 2 with stearic, oleic, linoleic, linolenic or arachidonic acids. Also one or two phytanic acid molecules have been introduced as acyl groups. The diglycerides were obtained by enzymic hydrolysis with phospholipase C from the respective phosphatidylethanolamines.

These synthetic phospholipids species, particularly those with polyunsaturated fatty acids, resemble closely those present in phospholipid mixtures from different biological membranes.

Using these well defined molecular species we studied the influence of the fatty acid hydrocarbon chains, of the hydrophilic part of the molecule and of the temperature on the stability and compressi-

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bility of their monomolecular films. The relevance of our results to membrane concepts is discussed.

Results and Discussion

Phospholipid monolayers of the following synthetic phosphoglycerides and diglycerides with the combinations of fatty acids listed below were studied:

1. Phosphatidylethanolamines

1,2-distearoyl-
1-stearoyl-2-oleoyl-
1,2-dioleoyl-
1-stearoyl-1-linoleoyl-
1-stearoyl-2-linolenoyl-
1-stearoyl-2-arachidonoyl-
1-stearoyl-2-phytanoyl-
1-phytanoyl-2-arachidonoyl-
1,2-diphytanoyl-

2. Phosphatidylcholines

1,2-distearoyl-
1-stearoyl-2-oleoyl-
1,2-dioleoyl-
1-stearoyl-2-linoleoyl-
1-stearoyl-2-linolenoyl-
1-stearoyl-2-arachidonoyl-

3. Diglycerides

1,2-distearoyl-
1-stearoyl-1-oleoyl-
1-stearoyl-2-linoleoyl-
1-stearoyl-2-linolenoyl-
1-stearoyl-2-arachidonoyl-

The F/A curves of the phospholipid monolayers were measured with a surface film balance of the LANGMUIR type, which continously recorded the F/A isotherm in the correct dyn/cm—Å²/molecule scale. This instrument (developed by SUCKER and MESKAT[22] also permits careful control and regulation of the subphase temperature. Our kinetic measurements of the film pressure as a function of the area per molecule at constant temperature yields isotherms, which may reflect several distinct states of the monolayers, their number and shape depending on the chemical structure of the film-forming molecules, the temperature and the properties of the subphase.

We determined for each molecular species listed before, at temperatures between 10 and 40°C, a) the states of the film, b) the area (Å²) per molecule, c) the minimum of the film compressibility, d) the energy of compression and e) the reversibility of the F/A isotherms. Film compressibility is defined by the term

$$C = -\frac{1}{A}\left(\frac{\mathrm{d}A}{\mathrm{d}F}\right)_T.$$

The minimum of the compressibility is reached at that point of the isotherm, at which its slope reaches a maximum. The energy of compression corresponds to the area under the isotherm ($E_m = F \cdot A$). Monomolecular films can be reversibly compressed and expanded. The rate of the compression (V_C) and expansion (V_E) influence the values for the energy of compression, the area per molecule and the reversibility. This is shown in Table 1 for 18:0/18:2-lecithin at 20.2°C as an example.

A high velocity of compression leads to an increased energy of compression, because the molecules of the film respond with a delayed orientation. Consequently an increase in the pressure at the collapse point and a decrease of the area per molecule can

Table 1. Influence of the rate of compression on the monolayer properties of 18:0/18:2-lecithin. V_C: Rate of compression; V_E: rate of expansion; A_M: area/molecule; R: reversibility (%); C_M: minimum of compressibility; E: molar energy of compression.

V_C [Å² · molecule^{-1} · min^{-1}]	V_E [Å² · molecule^{-1} · min^{-1}]	A_M [Å²]	R [%]	C_M [dyn/cm]	E [kcal/mole]
52.5	73.5	87.75	92.7	$1.10 \cdot 10^{-2}$	5.71
105	147	86.50	92.2	$1.10 \cdot 10^{-2}$	5.81
188	263	85.25	88.1	$0.92 \cdot 10^{-2}$	5.99
300	420	84.75	90.8	$0.88 \cdot 10^{-2}$	6.14

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be observed. All subsequent measurements were therefore carried out at constant velocities of compression ($V_C = 125\,Å^2 \cdot \text{molecule}^{-1} \cdot \text{min}^{-1}$) and expansion (175 $Å^2 \cdot \text{molecule}^{-1} \cdot \text{min}^{-1}$).

Influence of temperature on the states of phosphatidylethanolamine, phosphatidylcholine and diglyceride monolayers

The isotherms in the following Fig. 1, 2 and 3 of monolayers of some synthetic lipids evidently show that the state of the film is less dependent on the hydrophilic group of the molecule than upon the hydrophobic groups, namely the structure of the fatty acid residues.

Fig. 4, 5, 6 and 7 represent the F/A isotherms of 1-stearoyl-2-oleoyl glycerol at temperatures of 10, 20, 30 and 40°C. They indicate that the state of the monolayer is dependent on the temperature. The diglyceride film slowly passes over from the liquid expanded into the solid condensed state. Monolayers of 1,2-distearoyl glycerol and 1,2-distearoyl-3-glycerophosphoryl choline exhibit only the condensed state between 10°C and 40°C.

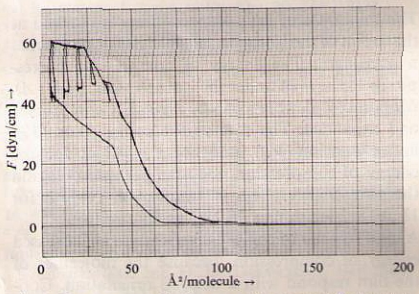

Fig. 1. Pressure-area isotherms of 1-stearoyl-2-oleoyl-3-glycerophosphorylethanolamine at 25°C.

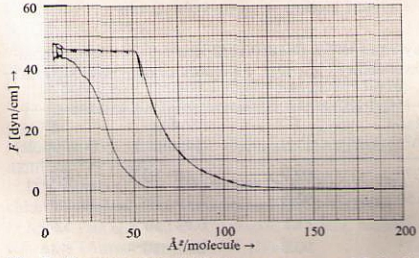

Fig. 2. Pressure-area isotherms of 1-stearoyl-2-linolenoyl-3-glycerophosphorylethanolamine at 25°C.

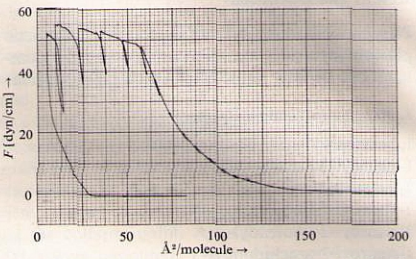

Fig. 3. Pressure-area isotherms of 1-stearoyl-2-linoleoyl-3-glycerophosphorylcholine at 25°C.

The insertion of only one *cis*-double bond to yield 1-stearoyl-2-oleoylglycerol and the respective phosphatidylcholine and -ethanolamine leads to the diglyceride and phosphatidylethanolamine below 35°C (Fig. 5) and phosphatidylcholine below 10°C being in the condensed state. Whereas 1-stearoyl-2-oleoyl-3-glycerophosphorylethanolamine forms both the liquid expanded and the condensed states depending on the film pressure. However 1-stearoyl-2-linolenoyl-3-glycerophosphorylethanolamine and 1-stearoyl-2-linoleoyl-3-glycerophosphorylcholine remains in the liquid expanded state at all pressures up to the pressure at the collapse point. As a consequence of the change in the state of the monolayer the reversibility of the species within a lipid class becomes temperature dependent.

Compressibility of monolayer films

Two structural features of the phospholipid molecules essentially influence the compressibility of monomolecular films, namely the hydrophobic and the hydrophilic parts of the molecules.

We determined the minimum of the compressibility C_M of a monolayer by determining the slope of the isotherm at the point of inflection of the F/A isotherm, divided by the molecular area at the point of inflection. All our liquid expanded films had only one point of inflection, mostly close to the collapse point.

The following table indicates that the minimum of compressibility of the diglycerides and lecithins is distinctly larger than that of the cephalins (Table 2).

Species of molecules of the same phospholipid class with more than two *cis*-double bonds in the alkane chains possessed similar C_M values (Table 3 and 4).

However phytanoyl-2-arachidonoylphosphatidylethanolamine showed a strongly elevated C_M value at all temperatures at which it was measured. We assume that the CH_3-groups of the phytanoyl group

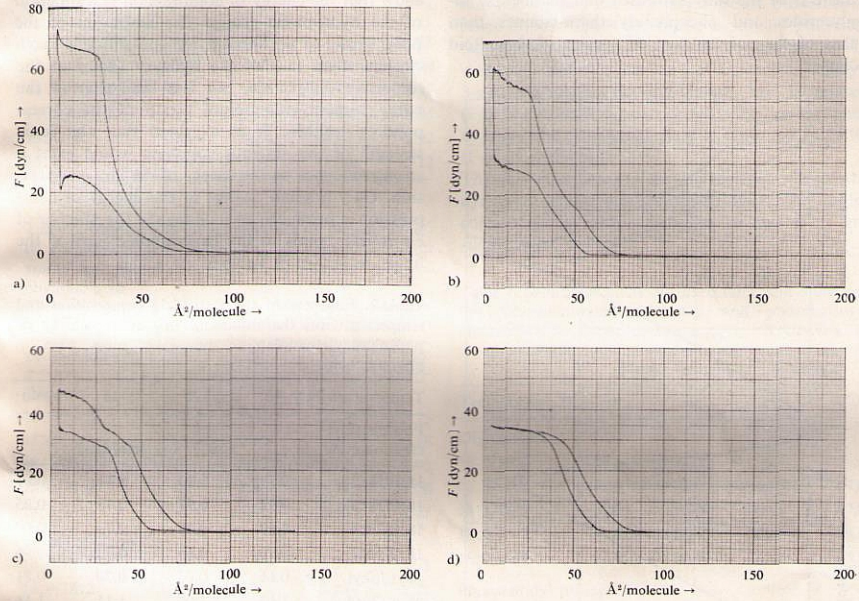

Fig. 4. Pressure-area isotherms of 1-stearoyl-2-oleoylglycerol at 10°C (a), 20°C (b), 30°C (c) and 40°C (d).

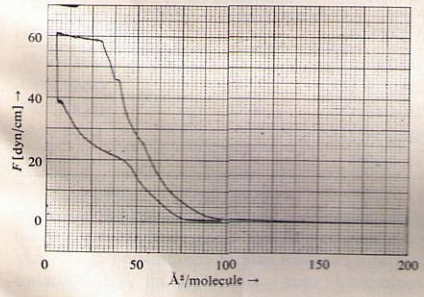

Fig. 5. Pressure-area isotherms at 20°C of 1-stearoyl-2-oleoyl-3-glycerophosphorylethanolamine.

Table 2. Influence of the hydrophilic group of the lipid molecule on C_M at 10, 20 and 30°C. DG = diglyceride; PC = phosphatidylcholine; PE = phosphatidylethanolamine.

Temperature [°C]	10	20	30
Compound		$10^{-2} \cdot C_M$ [cm/dyn]	
18:0/18:2 DG	1,35	0.93	0.97
18:0/18:2 PC	0.77	0.97	1.14
18:0/18:2 PE	0.73	0.80	0.81
18:0/18:3 PC	1.06	0.88	1.04
18:0/18:3 PE	0.92	0.77	0.78
18:0/20:4 DG	1.30	1.35	1.38
18:0/20:4 PE	0.67	0.76	0.79

fit into the space of the *cis*-double bonds of the neighbouring arachidonoyl residue. Table 3 also indicates that C_M is barely influenced by the temperature as long as the monolayer remains in the same state. The temperature more strongly affects the C_M of the fully saturated and monoenoic diglycerides and phosphatidylethanolamines than that of the polyunsaturated species of these lipid classes.

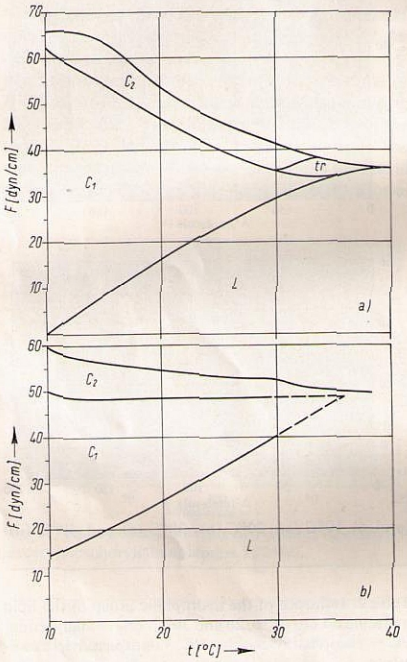

Fig. 6. Film pressure-temperature phase diagram of a) 1-stearoyl-2-oleoylglycerol; b) 1-stearoyl-2-oleoyl-3-glycerophosphorylethanolamine.

Energy of compression

Our experimental facilities also permitted the determination of *energy of compression*, which again is dependent on both the hydrophilic and lipophilic groups. Monolayers of a diglyceride, phosphatidylethanolamine and -choline with identical fatty acid composition (1-stearoyl-2-oleoyl—and 1,2-dioleoyl) were compressed from 250 Å²/molecule to 5 Å²/molecule at a constant rate of compression (125 Å² · molecule^{-1} · min^{-1}). These three types of molecules differ only in the structure of the polar groups and the degree of their hydration. The results in Table 5 show that the energy of compression is a function of the hydrophilic group. The hydration of the polar group of phosphatidylethanolamine is much stronger than that of the hydroxy group of the respective diglyceride, but less than that of the bulky trimethylammonium group of the corresponding lecithin. It is expected that the ionic strength of the aqueous subphase would exert a strong influence on the energy of the film compression. Only a small change in the energy of compression is produced by a change in the number of *cis*-double bonds in the hydrophobic part of the lipids, as shown in Table 6.

Table 3. Influence of the fatty acid composition and temperature on the minimum of compressibility of phosphatidylethanolamine monolayer films. phyt. = phytanic acid.

Temperature [°C]	10	20	30	40
Phosphatidyl-ethanolamine		$10^{-2} \cdot C_M$ [cm/dyn]		
18:0/18:2	0.73	0.80	0.81	0.97
18:0/18:3	0.92	0.77	0.78	0.85
18:0/20:4	0.67	0.76	0.79	0.90
18:0/phyt.	0.70	0.65	0.73	0.96
phyt./phyt.	0.66	0.65	0.70	0.81
phyt./20:4	1.08	1.00	1.11	1.14

Table 4. Influence of the fatty acid composition on C_M of diglyceride monolayers at 10°C.

Diglyceride	$10^{-2} \cdot C_M$ [cm/dyn]
18:0/18:0	0.27
18:0/18:1	0.46
18:1/18:1	0.87
18:0/18:2	1.35
18:0/20:4	1.30

The *collapse pressure* depends on the velocity of the compression. All our measurements were carried out at a constant rate of compression of 125 Å² · molecule^{-1} · min^{-1}. Therefore all F/A isotherms were recorded at above the equilibrium spreading pressure. The conditions however permitted a bet-

Table 5. Influence of the polar hydrophilic group of the lipid molecule on the energy (E) of film compression. DG = diglyceride; PE = phosphatidylethanolamine; PC = phosphatidylcholine.

	E [kcal · mole^{-1}]			
Temperature [°C]	10	20	30	40
Compounds				
18:0/18:1 DG	3.35	2.96	2.68	2.55
18:0/18:1 PE	4.25	4.07	4.04	3.90
18:0/18:1 PC	5.19	5.15	5.07	4.95
18:1/18:1 DG	3.31	3.27	3.14	2.95
18:1/18:1 PE	4.25	4.14	4.03	3.92
18:1/18:1 PC	5.35	5.31	5.28	5.12

Table 6. Influence of the temperature on the energy (E) of compression of diglyceride, phosphatidylethanolamine and phosphatidylcholine monolayers. phyt. = phytanic acid.

	E [kcal · mole^{-1}]			
Temperature [°C]	10	20	30	40
Diglyceride				
18:0/18:0	3.83	3.45	3.34	3.25
18:0/18:1	3.35	2.96	2.68	2.55
18:1/18:1	3.31	3.27	3.14	2.95
18:0/18:2	4.13	3.80	3.74	3.76
18:0/20:4	3.80	3.61	3.40	3.47
Phosphaditylcholine				
18:0/18:1	5.19	5.15	5.07	4.95
18:0/18:2	5.76	5.58	5.28	5.12
18:0/18:3	5.51	5.09	4.99	4.91
Phosphatidylethanolamine				
18:0/18:1	4.25	4.07	4.04	3.90
18:0/18:2	4.76	4.42	4.25	4.08
18:0/18:3	4.89	4.52	4.35	4.12
18:0/20:4	4.81	4.32	4.46	4.53
18:0/phyt.	5.15	4.99	5.04	4.63
phyt./20:4	5.57	5.55	5.35	5.09
phyt./phyt.	5.81	5.74	5.71	5.34

ter insight into the dynamics of the monolayer. All phosphatidylethanolamine, phosphatidylcholine and diglyceride molecules with polyunsaturated fatty acids or two oleic acid molecules only form monolayers of the liquid expanded state between 10 and 40°C and between 125 Å^2 · molecule^{-1} · min^{-1} and the collapse point.

The same is true for 1-phytanoyl-2-arachidonoyl-1-stearoyl-2-phytanoyl- and 1,2-diphytanoyl-3-glycerophosphorylethanolamine, although the latter two are fully saturated. Their alkyl chains cannot be compressed into the closest packing of a condensed film but instead the monolayers reach the collapse point directly from the liquid expanded phase. If the pressure/temperature-phase diagrams of 1-stearoyl-2-oleoylglycerol and of 1-stearoyl-2-oleoyl-3-glycerophosphorylethanolamine are compared (Fig. 6) a transition point between the liquid expanded and the condensed state is observed at 35°C (Fig. 6a and 6b).

The *molecular areas* of the diglycerides, phosphatidylethanolamine and phosphatidylcholine molecules at temperatures between 10 and 40°C are summarized in Table 7.

Table 7. Molecular areas of mixed fatty acid diglycerides, phosphatidylethanolamines and phosphatidylcholines at different temperatures. phyt. = phytanic acid.

	Molecular area [Å^2/molecule]			
Temperature [°C]	10	20	30	40
Diglycerides				
18:0/18:0	36.5	36.3	36.3	35.5
18:0/18:1	42.0	42.0	44.0	44.0
18:1/18:1	55.8	57.0	69.5	60.0
18:0/18:2	52.8	62.0	65.5	69.8
18:0/20:4	57.5	59.5	61.0	65.0
Phosphatidylcholines				
18:0/18:1	40.0	52.5	57.0	59.5
18:0/18:2	50.5	56.3	58.8	60.0
18:0/18:3	47.0	55.5	57.5	59.5
Phosphatidylethanolamines				
18:0/18:1	37.0	36.8	32.5	46.3
18:0/18:3	45.8	50.5	55.0	57.8
18:0/20:4	51.0	52.5	55.8	57.5
18:0/phyt.	57.5	58.0	61.3	57.0
phyt./phyt.	68.8	64.5	71.0	67.8
phyt./20:4	64.0	64.5	64.5	66.5

The molecular area at the collapse point of the lipids becomes larger with increasing temperature except in the case of 1,2-distearoylglycerol, which is present in the condensed state with the two stearic

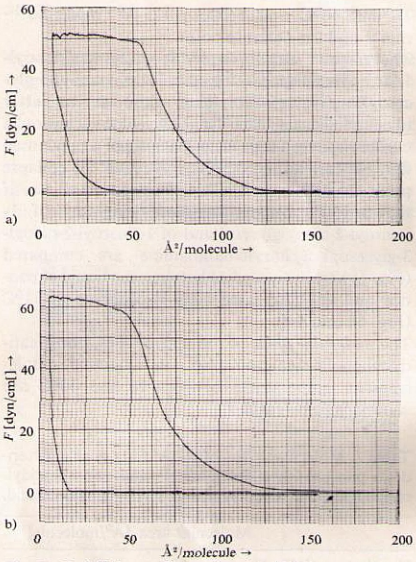

Fig. 7. Stabilizing effect of saturated phosphatidylcholine on the monolayer film of polyunsaturated phosphatidylcholine.
Pressure-area isotherms at 20°C of 1-stearoyl-2-linolenoyl-3-glycerophosphorylcholine monolayer film (a) and after the admixture of 3.5% of 1,2-distearoyl-3-glycerophosphorylcholine (b).

acids closely packed like a crystalline solid. 1-Stearoyl-2-oleoyl-3-glycerophosphorylethanolamine also forms a condensed monolayer if the temperature is lowered beyond the transition temperature. All the other lipid species, most prominently those with phytanic acid residues, reach the collapse point at very loose packing.
1,2-distearoyl diglyceride has a molecular area of 40—45 Å²/molecule while all other diglycerides, phosphatidylethanolamines and phosphatidylcholines investigated had between 70 and 90 Å²/molecule and the phytanic acid containing phosphatidylethanolamines had about 90—100 Å²/molecule.
The collapse pressure is strongly influenced by the polar group of the lipids. It is highest for phosphatidylcholine followed by phosphatidylethanolamine and then the diglyceride. In general it can be seen from our experiments that the collapse pressure of the condensed monolayers exceeds that of films which reach the collapse point from their liquid expanded state directly, by 25—50%.
Monolayers of unsaturated phospholipids are effectively stabilized if even a small percentage of saturated lipids is built into the film. Figures 7a and 7b compare F/A isotherms at 20°C of the monolayer of 1-stearoyl-2-linolenoyl-3-glycerophosphoryl choline without and with the admixture of 3.5% distearoyl-phosphatidylcholine. The collapse pressure was raised by 10 dyn/cm.

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Experimental

Materials

Optically pure L-α-cephalins and L-α-lecithins with a well defined fatty acid distribution were synthesized by methods described essentially by DAEMEN *et al.*[23,24] and DE HAAS and VAN DEENEN[25]. The 1,2-diacylglycerols were prepared from the corresponding phospholipids by enzymic hydrolysis with phospholipase C from *Bacillus cereus*[26]. They were purified by the method of BLIGH and DYER[27]. The purity of the phospholipids was checked by thin-layer chromatography in the system: chloroform/methanol/water 65:35:4 (v/v), that of the diglycerides in the system: petroleum ether/ether/acetic acid 90:10:1.5 (v/v). The fatty acid distribution of the synthetic lipids in position 1 and 2 was checked in the following way: the fatty acid in position 2 was enzymically released by phospholipase A_2 from *Crotalus adamanteus*, those in position 1 were obtained by treatment of the lysophosphatides with hydrochloric acid in methanol. The analysis of the fatty acids was carried out as their methyl esters by gas liquid chromatography.

Determination of force-area-isotherms

The force-area-isotherms were recorded with a LANGMUIR surface balance, constructed by Dr. CHR. SUCKER, Farbenfabriken Bayer AG, Leverkusen. The trough and

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all parts of the balance which contacted the aqueous phase or the monomolecular film were coated with teflon. The deflection of the force sensitive barrier was only 10^{-4} mm · dyn^{-1} · cm^{-1} and therefore did not need to be compensated. The film compressing barrier was driven by an electric motor, which allowed the measurement and recording of the force-area-isotherms automatically and continously within 3 minutes.

The measurements were carried out with aliquots of solutions of the lipids in benzene, which were spread on the water surface. The solvent was allowed to evaporate for 3 min before compressing the films. Water redistilled from a quartz still was used; the pH was about 5.8. The solvent used was benzene Merck, p. a., $nD^{20} = 1.5005$ to 1.5015. All measurements were carried out with a compression rate of 125 Å^2 · molecule^{-1} · min^{-1} and an expansion rate of 175 Å^2 · molecule^{-1} · min^{-1}. The initial available surface was about 400 Å^2/molecule and the force-area-isotherms were recorded between 250 Å^2/molecule and 5 Å^2/molecule. The temperature of the substrate could be varied between 10°C and 40°C. The room temperature was held constant at 20°C.