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SURFACE PROPERTIES OF SYNTHETIC PHOSPHOLIPIDS

II. THERMAL PHASE TRANSITIONS IN MONOLAYERS

MAKOTO HAYASHI, TOSHIO MURAMATSU and ICHIRO HARA

Laboratory of Chemistry, The Department of General Education, Chiba University, Chiba, and Laboratory of Chemistry, The Department of General Education, Tokyo Medical and Dental University, Ichikawa (Japan)

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SUMMARY

1. Thermal phase transitions were detected in monolayers of synthetic phospholipids by surface viscosity measurements.

2. The following transition temperatures were found for pure films: 35.5 °C for dipalmitoylphosphatidylethanolamine, 31 °C for dipalmitoylphosphatidyl-*n*-propanolamine, 29 °C for dipalmitoylphosphatidyl-*i*-propanolamine, and 8 °C for dipalmitoylphosphatidylcholine.

3. In mixed films of each of three phospholipids consisting of primary amine and dipalmitoylphosphatidylcholine, the transition temperatures are lowered as the proportions of the latter are increased, according to the law of melting point depression. The results were considered to show that they were separated into two phases in the solid films.

4. In mixed films of primary amine-containing phospholipids only, it is inferred that they exist in a homogeneous single phase even in the solid films.

5. It was deduced that the choline group cannot be introduced into the two-dimensional ionic lattice formed by primary amine and phosphate, because of a steric hindrance caused by their different end group sizes.

INTRODUCTION

The conformation and the orientation of phospholipids in membrane states have been focussed upon in order to study their basic properties in connection with their biological or physiological functions. However, further studies are still awaited to arrive at a concrete conclusion regarding this problem. We have studied synthetic phospholipids in a monolayer state, and a network structure was predicted for the monolayers of these compounds under the appropriate conditions[1]. This structure depends largely on the temperature at which the monolayer is observed, and also on the film compositions in mixed films. For example, dipalmitoylphosphatidylethanolamine gives an extremely rigid and contracted film on water when it is observed at ordinary room temperature. When the temperature is raised above 35.5 °C, however, it gives a mobile film, the pressure–area curve is largely expanded, and a definite transition temperature can be observed between the two states. These temperatures are specific to the respective systems not only in the pure state, but also in the mixed films. This fact is considered to demonstrate a critical change in state of the monolayer at this temperature. These temperatures were measured for five synthetic phospholipids, palmitic acid and cholesterol films, as well as their various combinations as mixed films. Then the conformations and the states of the film materials in monolayer were discussed in relation to the results obtained.

MATERIALS

Synthetic dipalmitoyl phospholipids used in this study are the same as those noted in the previous paper[1]. Palmitic acid was purified from a commercial reagent by fractional distillation under reduced pressure. Cholesterol was purified by preparative thin-layer chromatography from a commercial reagent, and was confirmed to give a single spot on thin-layer plate. NaCl was roasted at about 700 °C for 3 h to remove greasy materials. Other inorganic reagents were treated with activated charcoal before use to remove surface active impurities. Organic solvents were purified in conventional ways for each preparation, respectively. Water was distilled twice over alkaline permanganate.

PRINCIPLE AND APPARATUS

The rheological natures of the monolayers may be used for the observation of their states[2], the principle of which was applied to the systems as follows: At a sufficiently low temperature, the film is extremely rigid and the surface viscosity cannot be detected by the canal method. When the temperature of the trough is elevated and reaches a definite point, the film is abruptly softened and the surface viscosity becomes observable. Thus, a characteristic temperature in this way is given for the respective films. To find out these temperatures, the following apparatus was used (the main part of it is identical to that noted in the previous paper[1]). Surface pressure changes are detected by an electromicrobalance combined with a hanging glass plate, the output signals from which are fed to a switching circuit for the barrier-driving motor so as to retain the surface pressure at a definite value. In this way, the rate of flow of the monolayer through the canal can be detected by the movement of the barrier. A platinum wire of about 2.5 m length was placed in the plane of the bottom of the trough as a heater by which the temperature in the trough was elevated. The platinum wire was uniformly spread over the whole area of the bottom, and alternating current was passed at 10 W to elevate the temperature at the rate of about 0.5 °C per min.

PROCEDURE FOR MEASUREMENT

First, the monolayer is made at a sufficiently low temperature (usually about 10 °C below the respective characteristic ones), under which condition it shows the behaviour of a highly viscous film such as calcium stearate or protein monolayers. At this temperature the film cannot flow through the canal of 1 mm width. After a stable surface pressure has been established (usually within several minutes), the shutter attached to the canal is taken out, and heating is started at the same time by feeding an electric current to the platinum wire in the trough. Thereafter, the reading of the temperatures for the time elapsed and the recording of the rate of flow of the monolayer, that is, the movement of the barrier, are simultaneously started, and both are recorded on a X–t recorder. An example of the recorded figures is shown in Fig. 1, in which it is seen that dipalmitoylphosphatidylethanolamine gives its characteristic temperature at 35.5 °C. In this figure, the horizontal line in the left-hand side of the curve shows that the film does not flow out through the canal, that is, the film is extremely viscous. In the right-hand side of the inflection point (35.5 °C), the curve declines markedly, which indicates the low viscosity of the film. This falling off of the curve indicates that the surface viscosity is as low as that of palmitic acid films at the same surface pressure on water.

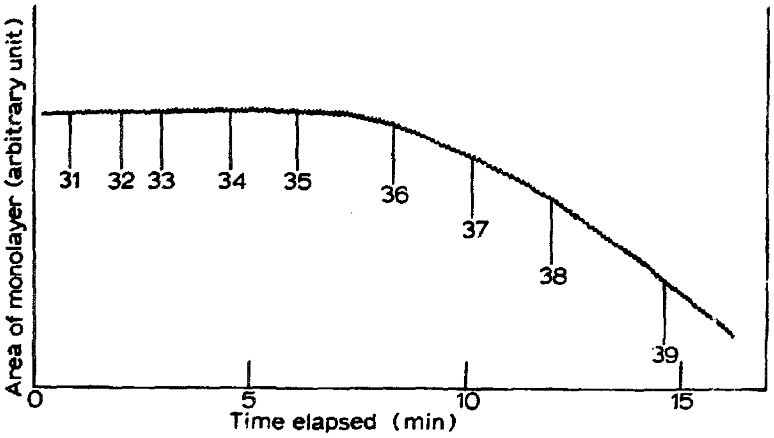
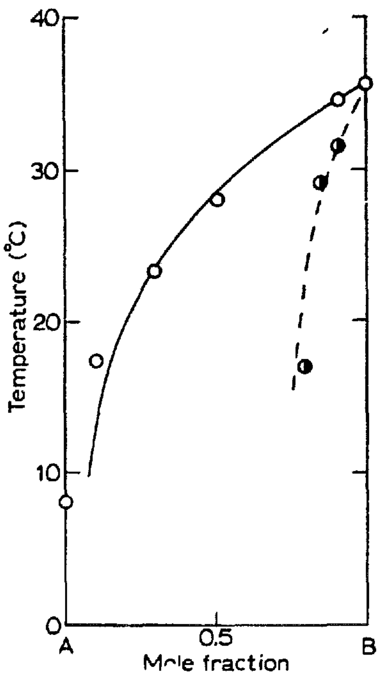


Fig. 1. Change in flow rate *versus* temperature in dipalmitoylphosphatidylethanolamine monolayer. Surface pressure, 10 dynes/cm; subphase solution, 0.1 M NaCl buffered at pH 5.5; ordinate, time elapsed after threshold of temperature raising; abscissa, area of the monolayer (arbitrary unit). The horizontal part of the curve shows zero flow rate. The numbers in the figure are temperatures in the trough (°C).

A chloroform–methanol (8:2, v/v) mixture was used as a spreading solvent for all lipids examined. The monolayers were spread by delivering 20–50 μl of spreading solutions onto the subphase surface using micropipettes. The characteristic temperatures described here were measured at 10 dynes/cm of surface pressure for the films of dipalmitoylphosphatidylethanolamine, dipalmitoylphosphatidyl-*n*-propanolamine, dipalmitoylphosphatidyl-*i*-propanolamine, dipalmitoylphosphatidylcholine, dipalmitoylphosphatidic acid and their mixtures in various combinations and compositions, and for the mixtures with palmitic acid and with cholesterol. Subphase solutions were made of 0.1 M NaCl and 0.01 M acetate buffer solution of pH 5.5. When palmitic acid was concerned, EDTA was added to this subphase solution at 0.001 M for masking heavy metal ions and so to prevent producing polyvalent metal palmitate.

RESULTS AND DISCUSSION

Figs 2–4 show the relationships between the compositions of the mixed films and their characteristic temperatures for various combinations. In these figures it is



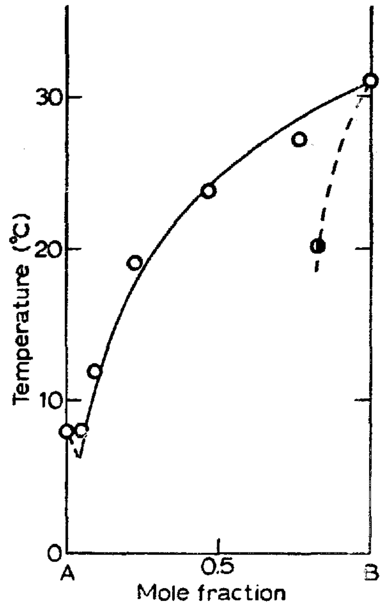
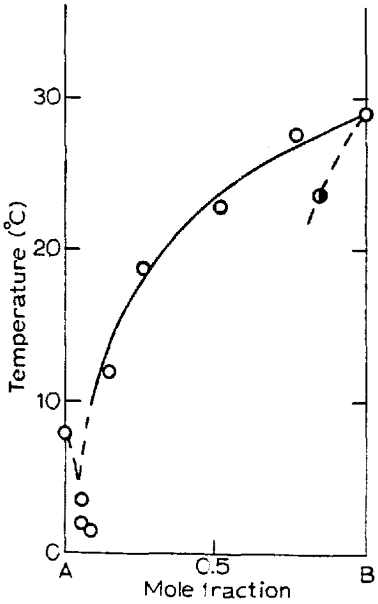


Fig. 2. Transition temperatures of the mixed films of dipalmitoylphosphatidylethanolamine (Component B) and dipalmitoylphosphatidylcholine (○) or cholesterol (◑) (Component A). Solid line, calculated curve (see text); subphase solution, 0.1 M NaCl buffered at pH 5.5, surface pressure, 10 dynes/cm.

Fig. 3. Transition temperatures of the mixed films of dipalmitoylphosphatidyl-*n*-propanolamine (Component B) and dipalmitoylphosphatidylcholine (○) or cholesterol (◑) (Component A). Solid line, calculated curve; conditions, see Fig. 2.



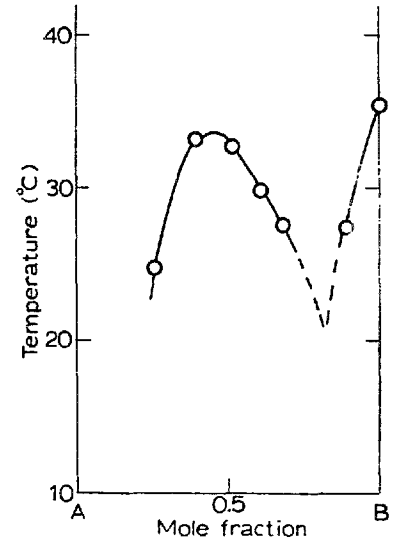


Fig. 4. Transition temperatures of the mixed films of dipalmitoylphosphatidyl-*i*-propanolamine (Component B) and dipalmitoylphosphatidylcholine (○) or cholesterol (◑) (Component A). Solid line, calculated curve; conditions, see Fig. 2.

Fig. 5. Transition temperatures of the mixed films of dipalmitoylphosphatidylethanolamine (Component B) and palmitic acid (Component A). Conditions, see Fig. 2.

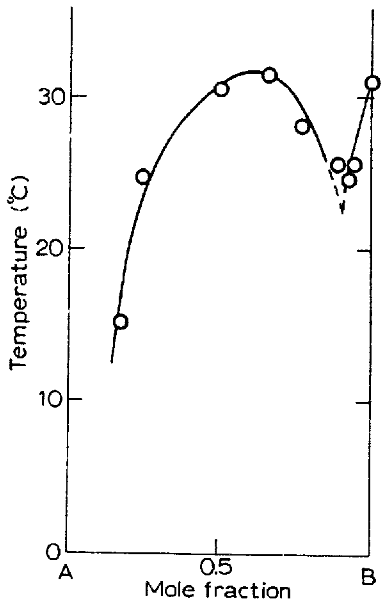
seen that dipalmitoylphosphatidylethanolamine, dipalmitoylphosphatidyl-*n*-propanolamine and dipalmitoylphosphatidyl-*i*-propanolamine in pure state are changed from the solid to the liquid states in the monolayers at 35.5 °C, 31 °C and 29 °C, respectively, and that they are lowered as the dipalmitoylphosphatidylcholine contents are increased in the mixed films. Cholesterol also has an effect similar to dipalmitoylphosphatidylcholine, but it is much stronger relative to the latter as seen in these figures.

Phillips and Chapman[3] deduced from their measurements of the temperature dependence of π–A curves that the two-dimensional phase transition observed in the monolayer of dipalmitoyllecithin was ascribed to a change in hydration in the choline moiety, and corresponded to a transition to a smectic structure from a crystalline state caused by hydration of polar moiety. Such thermal phase transitions in hydrated solid states have been observed in many studies for phospholipids[4–18], in all of which a penetration of water between polar groups plays an important role. The characteristic temperatures observed in our study, as defined in the previous section, might be considered to show the thermal phase transitions in monolayer state, that is to say, two-dimensional melting phenomena, which are likely to correspond to those cited above. If such is the case, the observed effects of mixing on the temperatures may be explained by the idea that they correspond to a melting point depression in monolayer state. In this point of view, these effects may be calculated by the following expression[19], provided that there is immiscibility of the components in the mixed films in solid state and perfect surface solution in liquid state:

$$-\ln x = (\Delta h^0/R)\,(1/T - 1/T^0) \qquad (1)$$

where x is the mole fraction of one component in the respective binary systems, Δh^0, T^0 represent enthalpy and temperature of melting in pure state of the respective films, and T is the melting point of the mixed film. Using Eqn 1, the melting point depression curves can be traced on the phase diagrams, if the heats of melting are tentatively estimated so as to follow the measured data. They are shown by the solid lines in Figs 2–4. In these figures, it can be said that the measured data sufficiently follow the calculated curves within the experimental error, which is derived from Fig. 1.

In view of these results, it may be postulated that each component in the mixed films examined here gives an ideal surface solution with the counter component, dipalmitoylphosphatidylcholine, in the liquid surface phases, and that they exist in the different phases in the solid states at the lower temperatures. These heterogeneous states in the solid phases lead to an inference that there must be invariant points on the phase diagrams, that is, eutectic points. In this case, these points must be close to the dipalmitoylphosphatidylcholine axes and below its melting point, 8 °C. Unfortunately, however, it is technically difficult to observe them clearly enough, since for this purpose, the observation must be started from near zero degrees as seen in the diagrams. Nevertheless, they were undoubtedly observed as shown in the cases of Figs 3 and 4. The eutectic phenomena are more distinctly found in the mixed films with palmitic acid, as seen in Figs 5 and 6. In these figures, however, the large deviations from an ideal behaviour are clearly seen in both cases, indicating strong interactions between palmitic acid and these phospholipids. The large deviation from the ideal is also seen in the mixed films with cholesterol, reflecting a marked interaction between cholesterol and the phospholipids, which has been predicted in many studies[20–27]. From the fact that all phospholipids give highly rigid monolayers, it can be concluded that they do form polymeric structures under appropriate conditions, which leads to the conception of the two-dimensional melting phenomena discussed above. It is probable that this structure consists of an intermolecular salt linkage between phosphate and amine in primary amine compounds, or quaternary ammonium ion in phosphatidylcholine. This is a kind of two-dimensional ionic lattice. It should be noted that the mixed monolayers are in a heterogeneous state consisting of two phases in solid state for all the instances. This fact indicates that phosphatidylcholine cannot be introduced into the lattice which consists of phosphate and primary amines such as that in phosphatidylethanolamine, as well as cholesterol or palmitic acid. This also may be inferred from the consideration that these two groups, primary amine and quaternary ammonium ions, are largely different from each other in their dimensions. These points of view strongly support an idea that they separately exist in the different structures of two-dimensional lattice in the mixed monolayers at sufficiently low temperatures. When the temperature of the mixed monolayer is raised, and arrives at the eutectic point, the lower melting point component must be melted as an eutectic mixture. At this point, however, the fluidity of the mixed monolayer itself cannot yet be observed, since the rest of the higher melting point component is still in the network structure made of itself, and the former is trapped in it. Thereafter, when arriving at the melting point of the mixed film, the whole monolayer is completely melted and becomes mobile.



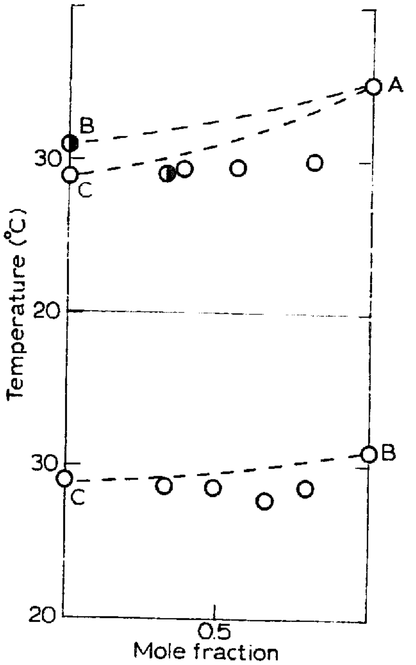


Fig. 6. Transition temperatures of the mixed films of dipalmitoylphosphatidyl-*n*-propanolamine (Component B) and palmitic acid (Component A). Conditions, see Fig. 2.

Fig. 7. Transition temperatures of the mixed films. Component A, dipalmitoylphosphatidylethanolamine; Component B, dipalmitoylphosphatidyl-*n*-propanolamine; Component C, dipalmitoylphosphatidyl-*i*-propanolamine; broken lines, calculated curves (see text); conditions, see Fig. 2.

On the other hand, the mixed films between the primary amine-containing phospholipids only have quite different natures in their surface behavior from those consisting of the primary amine and the quaternary ammonium ion mentioned above. The results are shown in Fig. 7. In these mixed films, there no longer appears such an invariant system as the eutectic point. Only the continuous curves are given in the phase diagrams as mono-variant systems. The broken lines in these diagrams are the calculated curves for the solids, according to the following equation, with the same heats of melting as those in the cases of Eqn 1, assuming an ideality for mixing:

$$\begin{gathered} x_2 = (e^{\lambda_1} - 1)/(e^{\lambda_1+\lambda_2} - 1) \\ \lambda_1 = (\Delta h_1{}^0/R)(1/T - 1/T_1{}^0),\ \lambda_2 = (\Delta h_2{}^0/R)(1/T_2{}^0 - 1/T) \end{gathered} \tag{2}$$

where x_2 is the mole fraction of Component 2 in solid surface phase, and the subscrips 1 and 2 refer to Components 1 and 2. These results indicate that the mixing can occur in a single surface lattice even in the solid state, when different primary amine-containing phospholipids are mixed together, and the mixed films behave as a single phase in the solid state, and also indicate that the slight differences in structure among the polar moieties except the end group do not interrupt each other for forming a mixed lattice. This consideration leads to the conclusion that the films of these compounds consist of the intermolecular salt linkages formed between the phosphate and amine groups in different molecules at sufficiently low temperature. They are made up of adjacent molecules in two-dimensional lattice form between either the same or different molecules. It also leads to the conclusion that an additional methylene lying between the phosphate and amine groups is not a serious factor as a steric hindrance for forming this structure, and neither is a methyl side chain attached to this moiety.

In contrast to this, it is worth noting that the dimensions of the end groups (amine groups and quaternary ammonium ions) give a strong effect on the lattice formation. Dipalmitoylphosphatidylcholine has lower surface melting point (8 °C), which is far from that of phosphatidylethanolamine (35.5 °C), because of the larger ionic radius of the quaternary ammonium ion in the former, where an enhanced hydration may also be included. Further, these two kinds of polar end groups cannot co-exist in a single lattice, and they are completely separated by this difference in the solid state.

At temperatures above the two-dimensional melting points, however, this effect of the end groups on mixing disappears, and the mixing comes to be entropic between primary amine and quaternary ammonium ion compounds, and the surface viscosities are all very low. Putting these facts together, it is considered that the polar moiety of each component molecule would be in an intramolecular discharged form between ionogenic groups in all the cases under these conditions. This structure is identical to that deduced by Shah and Schulman[28,29] for dipalmitoylphosphatidylcholine monolayers.

We observed that monopalmitoylphosphorylcholine was very soluble in water and dispersed in a molecular dispersion in dilute solution[30], but it gave no electric conductance at all. This fact supports the above idea concerning the conformation of phosphatidylcholine moiety in monolayer under the appropriate conditions.

Latent heats and entropies of melting obtained from the above argument are given as 18.3, 20.1, 21.3 kcal/mole, and 59, 66, 70 cal/degree per mole for pure films of dipalmitoylphosphatidylethanolamine, dipalmitoylphosphatidyl-*n*-propanolamine and dipalmitoylphosphatidyl-*i*-propanolamine, respectively, which have been estimated so as to follow the given data. These values are considerably high relative to those for the usual three-dimensional ionic lattices. This might reflect the strong structures in the solid films of these compounds.

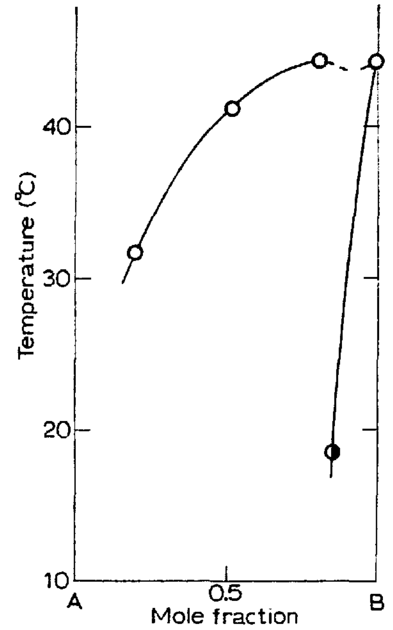


Fig. 8. Transition temperatures of the mixed films of dipalmitoylphosphatidic acid (Component B) and dipalmitoylphosphatidylcholine (○) or cholesterol (◑) (Component A). Conditions, see Fig. 2.

Dipalmitoylphosphatidic acid also gives a rigid film at room temperature, which superficially closely resembles the other phospholipids examined in this study. The two-dimensional melting point is 45 °C in pure film, and it is also lowered by mixing cholesterol or dipalmitoylphosphatidyl choline in it. The result is shown in Fig. 8. It is, therefore, inferred that there also must be a polymeric structure in this film. However, the linkage existing in this film should be inherently different from the others because of lack of cationic group. In monooctadecylphosphate monolayers, a polymeric structure was deduced by a surface viscosity measurement, in which hydrogen bonding between phosphates of adjacent molecules was considered to dominate this structure[31,32]. Referring to this result, the same structure will be probable in a phosphatidic acid film. This is also disrupted by mixing cholesterol or dipalmitoylphosphatidylcholine into the film as mentioned above. This may be explained in the same way as those in other cases. The marked departures from ideality of mixing in Fig. 8 suggest again interactions between the constituents in the mixed films.

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REFERENCES

1 Hayashi, M., Muramatsu, T. and Hara, I. (1972) *Biochim. Biophys. Acta* 225, 98
2 Deamer, D. W. and Cornwell, D. G. (1966) *Biochim. Biophys. Acta* 116, 555
3 Phillips, M. C. and Chapman, D. (1968) *Biochim. Biophys. Acta* 163, 301
4 Chapman, D. and Salsbury, N. J. (1966) *Trans. Faraday Soc.* 62, 2607
5 Chapman, D., Byrne, P. and Shipley, G. G. (1966) *Proc. R. Soc. London Ser. A 290*, 115
6 Chapman, D., Williams, R. M. and Ladbrooke, B. E. (1967) *Chem. Phys. Lipids* 1, 445
7 Bangham, A. D. (1968) in *Progress in Biophysics and Molecular Biology* (Butler, J. A. V. and Noble, D., eds), Vol. 18, p. 33, Pergamon Press, Oxford, New York
8 Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., ed.), p. 104, Academic Press, New York and London
9 Salsbury, N. J. and Chapman, D. (1968) *Biochim. Biophys. Acta* 163, 314
10 Ladbrooke, B. D. and Chapman, D. (1969) *Chem. Phys. Lipids* 3, 304
11 Abramson, M. B. (1970) *J. Colloid Interface Sci.* 34, 571
12 Williams, R. M. and Chapman, D. (1970) in *Progress in the Chemistry of Fats and Other Lipids* (Holman, R. T., ed.), Vol. 11, Part I, p. 11, Pergamon Press, Oxford, New York
13 Melchior, D. L., Morowitz, H. J., Sturtevant, J. M. and Tian Yow Tsong (1970) *Biochim. Biophys. Acta* 219, 114
14 Abramson, M. B. (1970) in *Surface Chemistry of Biological Systems* (Blank, M., ed.), p. 37, Plenum Press, New York, London
15 Trauble, H. (1971) *Naturwissenschaften* 58, 277
16 Trauble, H. and Haynes, D. H. (1971) *Chem. Phys. Lipids* 7, 324
17 Rand, R. P., Tinker, D. O. and Fast, P. G. (1971) *Chem. Phys. Lipids* 6, 333
18 Abramson, M. B. (1971) *Biochim. Biophys. Acta* 225, 167
19 Prigogine, I., Defay, R. and Everett, D. H. (1969) *Chemical Thermodynamics*, p. 357, Longmans, London
20 DeBernard, L. (1958) *Bull. Soc. Chim. Biol.* 40, 161
21 Dervichian, D. G. (1964) in *Progress in Biophysics and Molecular Biology* (Butler, J. A. V. and Huxley, H. E., eds), Vol. 14, p. 316, Pergamon Press, Oxford and New York
22 Shah, D. O. and Schulman, J. H. (1967) *J. Lipid Res.* 8, 215
23 Demel, R. A., van Deenen, L. L. M. and Pethica, B. A. (1967) *Biochim. Biophys. Acta* 135, 11
24 Zull, J. E., Greanoff, S. and Adam, H. K. (1968) *Biochemistry* 7, 4172
25 Demel, R. A. and Joos, P. (1968) *Chem. Phys. Lipids* 2, 35
26 Lecuyer, H. and Dervichian, D. G. (1969) *J. Mol. Biol.* 45, 39
27 Kamel, A. M., Felmeister, A. and Weiner, N. D. (1971) *J. Lipid Res.* 12, 155
28 Shah, D. O. and Schulman, J. H. (1967) *J. Lipid Res.* 8, 227
29 Shah, D. O. (1970) in *Advances in Lipid Research* (Paoletti, R. and Kritchevsky, D., eds), Vol. 8, p. 402, Academic Press, New York and London
30 Hayashi, M., Okazaki, M. and Hara, I. (1972) *6th Int. Congr. on Surface Active Substances, Zürich, 1972*
31 Gershfeld, N. L. and Pak, C. Y. C. (1967) *J. Colloid Interface Sci.* 23, 215
32 Muller, H., Friberg, S. and Hellsten, M. (1970) *J. Colloid Interface Sci.* 32, 132