Biochimica et Biophysica Acta, 307 (1973) 270–278

BBA 76314

RHEOLOGIC HYSTERESIS CURVE SHAPE SPECIFICITY OF MIXED LIPID FILMS

DEAN LUSTED

Department of Pathology, University of Texas, Southwestern Medical School at Dallas, Dallas, Texas 75235 (U.S.A.)

(Received November 24th, 1972)

SUMMARY

1. The surface tension (γ)–area (A) hysteresis loops of mixed lipid films were studied to discern if such a procedure might be suitable to investigate lipid domains and the effects of molar composition.

2. Using cyclic film compression and the Wilhelmy measurement of surface tension, it was shown that there is an evolution of both γ–A hysteresis curve shape and loop size.

3. With film composition being the only variant in the experimental system, the γ–A shape often was distinctive.

4. Specific zones of one hysteresis trace were interpreted as representing phospholipid ionic interaction.

5. Differential, sequential entry of the phospholipid acyl chains and polar groups in the hysterectic process indicated the separate importance of the forces governing the two parts of the film.

6. Controlled ionic concentration, pH, and temperature could effect an equalization of magnitude between the "strong" ionic forces of the polar groups and the "weak" dipole forces of the hydrocarbon chains.

7. With balanced forces functional significance of molar composition could be expressed through grouping of the lipids into interacting domains.

INTRODUCTION

In the fluid mosaic model of the biological membrane the lipid component is indicated as the matrix[1]. The physical state of these lipids would therefore be important both structurally and functionally. Two facts come to stand out: one, that lipids associate into domains; the other, that the biological membrane is differentially in the fluid phase with respect to locus.

The lipid domain concept is not new; grouping of lipid molecules was proposed by Warren[2] in 1933. Recent X-ray diffraction analysis of lipid bilayers has indicated the grouping of lipids into domains[3], while calorimetric studies indicate the presence of liquid crystals in *Mycoplasma laidlawii* membranes[4].

Fluidity in phospholipid bilayers has been demonstrated by spin-label assay in terms of lateral diffusion of phospholipids[5] and by the mobility of the phospholipid

hydrocarbon chains[6]. These studies of fluidity have been extended into biological membranes[7]. Cell fusion is another technique which has been used to quantitate membrane fluidity as viscosity[8].

Although the significance of the molar ratio of phospholipids has not been determined in biological membrane domain formation or fluidity, the importance of phospholipid composition to cellular organization and function is suggested by the molecular compositions unique for certain subcellular organelles performing specific functions. Substitution of one phospholipid for another in biological membranes with structure, and possibly function, remaining constant has been indicated[9]. An interesting parallel is that the importance of trace components and impurities in determining the structural properties of metal alloys through the modification of crystal structure has been established. Similar subtle composition changes in phospholipid composition might hold equivalent significance through altered properties of lipid interaction.

The investigation of lipid interactions was facilitated through the observations and hydrophil balance techniques developed by Rayleigh, Pockels, Langmuir, Harkins, and Adam. Leathes reported a "condensing effect" of cholesterol on fatty acid and lecithin films[10]. Similar studies with refinements in materials and instrumentation have been augmented by corresponding studies using differential scanning calorimetry and X-ray diffraction[11,12]. Cholesterol in a lipid film appears to be able to make an over-solid film more fluid or an over-fluid film more solid[11,13]. The enigma of the mechanism of action of cholesterol is compounded by its immiscibility with octadecanol, stearic acid, and dipalmitoyllecithin[14]. The observation that surface tension (γ)–film area (A) hysteresis characteristics changed according to the molar ratio of immiscible cholesterol to phospholipid suggested that lipid domains might be studied with the hydrophil balance[15].

The problem confronted was to discern the effect of molecular composition on the formation and interaction of lipid domains. Were a domain to be made up of 50 molecules, the experimental procedure should allow a time constant large enough for such a mass to respond. In the absence of "chemical interaction" would lipid interactions be demonstrable as rheologic hysteresis?

The reluctance shown by molecules to rearrangement may be quantitated as rheologic hysteresis. In the sense that "structure" resists deformation and that "active function" is associated with deformation, rheologic hysteresis might be considered the junction of structure and function. In this sense the greater the amount of hysteresis in molecular interactions the more "structural" the molecular configuration. That is to say that the configuration resists the potentially deforming force: a structure of blocks resists gravity: a structure of ordered magnetic domains resists a contrary magnetic field.

Since the demonstration by Langmuir[16] that the inclination of fatty acid molecules in a film at an air–water interface was related to surface pressure π, and by means of the formula,

$$\pi = \gamma_\omega - \gamma$$

where γ_ω represents the surface tension of clean water and γ represents the surface tension of water covered by a film giving surface pressure π, it is possible to assume

that surface tension also represents the positioning of the molecules in the film. Accordingly, an experiment was designed using a continuous, cyclic γ–A trace to indicate rheologic hysteresis.

MATERIALS AND METHODS

The apparatus designed to study the γ–A hysteresis properties of biological surfactants[17] was modified[15] from the commercially-available Cahn Surface Tension Accessory. The Teflon trough was uniformly filled with distilled water to edge level: the film area was limited by flat, Teflon, barrier blades riding on the 3-mm flat edges of the trough. Surface tension was determined by the Wilhelmy technique, using a roughened platinum plate[15].

The film area between the barrier blades ranged from 15.55 cm^2 to 34.56 cm^2, with a rate of 0.5 cycle/min, an increment of 15.8 Å/molecule per min. Although the film was performing far from equilibrium with this rate, it offered the possibility of a time constant possibly advantageous for the study of lipid domains.

A biological membrane being more complex than a monomolecular layer, the films in the experiment were mechanically compressed well past collapse. It was anticipated that this would exploit the formation and dispersion characteristics of the collapse structures that might form.

The hysteresis traces were considered comparable on the basis that the only variant in the experiment was the molecular composition of the lipid film[15].

Dipalmitoyllecithin and phosphatidylserine (Mann Research Laboratories), phosphatidylethanolamine (Applied Science Laboratories), phosphatidylinositol (Serdary Research Laboratories), sphingomyelin, and cholesterol (National Biochemical Corp.) were dissolved in *n*-hexane–ethanol (9:1, v/v) to make up an approximately $1 \cdot 10^{-3}$ M solution of each lipid. Combinations of these solutions were mixed volumetrically to establish the desired molar ratios in the mixed solutions. With the exception of synthetic lecithin, the acyl chain analysis was undetermined in these animal-origin lipids. The phospholipid mixture (Fig. 1) was of human origin and was composed of lecithin 29.5% phosphatidylethanolamine 23.2%, phosphatidylinositol 18.5%, lysolecithin 12.3%, lysophosphatidylethanolamine 9.2%, lysophosphatidylinositol 7.3%. No cholesterol or sphingomyelin was present.

20 μl of mixed solution were rapidly dispensed onto the clean, 25 °C, distilled water surface when the continuously cycling barrier blades were at maximum expansion (*i.e.* 20 μl of $1 \cdot 10^{-3}$ M lipid was spread onto 34.56 cm^2). Sequential numbering of the hysteresis loops was started with the first full compression of the film. The areas of these loops were determined with a planimeter. Whether or not to continue a test through the usual 40 min was based on agreement of the γ–A trace with established hysteresis loop size, shape, and usual sequence for that particular film.

RESULTS

In order to appreciate the problems and potential of this method Figs 1–6 are presented; (Fig. 1) to display the changing performance of a film and the reason for arbitrarily choosing one cycle for comparisons, (Fig. 2) to demonstrate the repli-

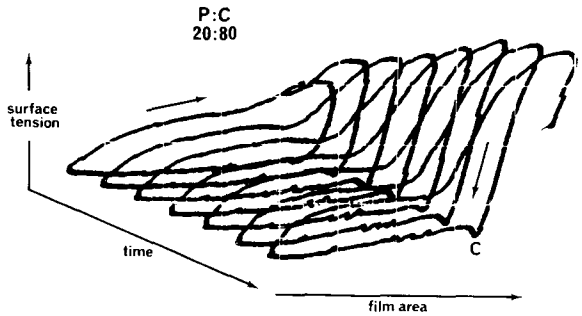

Fig. 1. Evolution of hysteresis loop size and shape. This continuous γ–A recording shows a progressive change in hysteresis loop size and shape. The film, composed of 20% mixed phospholipids (P) (see Materials and Methods) and 80% cholesterol (C), was spread from a hexane solution over 34.56 cm^2 of 25 °C distilled water between continuously cycling barriers. Notice that equivalent bucklings past the first point of film collapse (C) are surprisingly parallel to the time axis and indicate events intrinsic in the film, since no identical instrumental noise was present in other traces (not pictured).

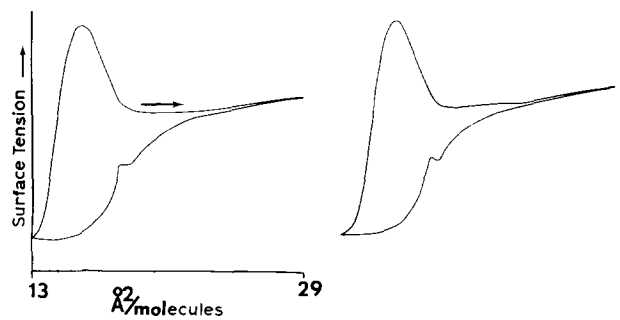

Fig. 2. Replicability of the γ–A loops. The third cycle of two films identically spread: 20 μl of the same $1 \cdot 10^{-3}$ M solution (20% dipalmitoyllecithin:20% phosphatidylserine:60% sphingomyelin in hexane–ethanol, 9:1, v/v) onto 25 °C water. The loop on the left had an area of 4.14 compared to 3.94 for that on the right. Shape often shows better replication than area in this method.

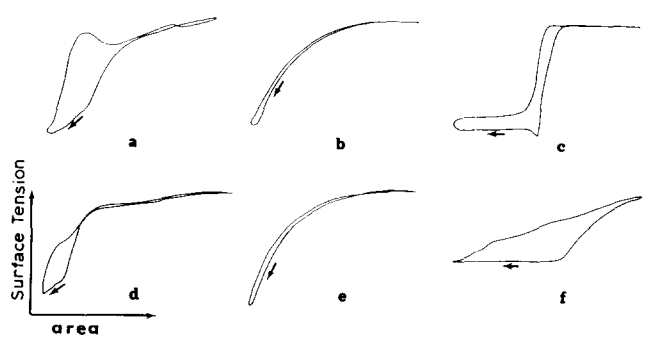

Fig. 3. Uniqueness of shape. Single molecular species of lipid can present recognizable features. Films of sphingomyelin (a), phosphatidylserine (b), cholesterol (c), dipalmitoyllecithin (d), phosphatidylinositol (e), and phosphatidylethanolamine (f) show different third-cycle traces. All the films were made from 20 μl of $1 \cdot 10^{-3}$ M lipid in hexane–ethanol (9:1, v/v) spread onto 25 °C distilled water.

cability of the method, (Fig. 3) to show the difference in γ–A shapes of one molecular species, (Fig. 4) to show both the effect of varying molar ratios and the difference in mixture shape from the component lipids, (Fig. 5) to give a sample interpretation of one trace, and (Fig. 6) to present a concept derivative from that interpretation.

One of the first things that became apparent with the use of this procedure was the fact that valuable information about the performance of a film might be lost, if one were to consider only the equilibrium trace in which there is close replication of sequential cycles. Fig. 1 illustrates the evolution of both size and shape in a continuous, time-offset, γ–A trace. Exact replication of performance is occasionally difficult, especially with certain films[15].

The hysteresis loop area was used to obtain the enumeration required for the

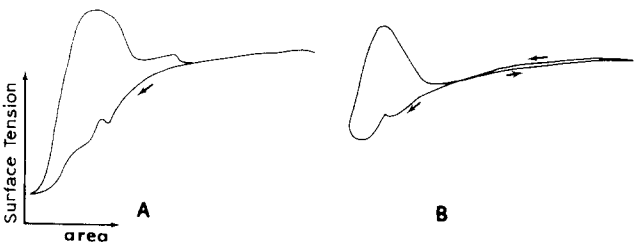

Fig. 4. Shape of mixed film different from components and variation with molar ratio. The molar ratio of component lipids in the films effects the shape. (A) is the γ–A shape from an 80% dipalmitoyllecithin:20% phosphatidylinositol film, whereas (B) is the shape from a 60% dipalmitoyllecithin:40% phosphatidylinositol film. Note the difference from Fig. 3d and 3e, the parent components. In each case 20 μl of $1 \cdot 10^{-3}$ M lipid in hexane–ethanol (9:1, v/v) was spread on 25 °C distilled water.

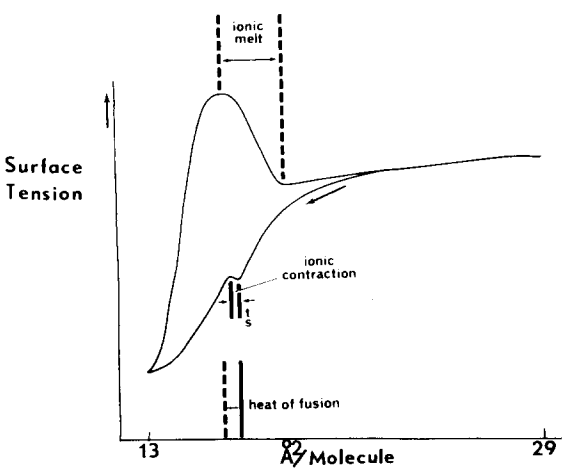

Fig. 5. A sample interpretation of a shape. Some shapes of mixed lipids which are different from the pure lipid shapes (*cf.* Figs 3b and 3d) suggest interaction of the components. There is a reason for believing that the new shape from 80% lecithin:20% phosphatidylserine is partially due to components based on the ionic composition of the interaction. The sequential responses of the types of bonding determines certain characteristics of the shape (see text). With a known rate of area change for such a trace an estimate could be made of domain mass from measuring time (t) and distance (s). The film was produced by spreading 20 μl of $1 \cdot 10^{-3}$ M 80% dipalmitoyllecithin: 20% phosphatidylserine premixed in hexane–ethanol (9:1, v/v) onto 25 °C distilled water.

statistical evaluation of the test replicability[15]. Hysteresis loop area, however, did not appear to be the most sensitive index of comparison. Fig. 2 illustrates a lack of area duplication, although there is a recognizable similarity in the shapes.

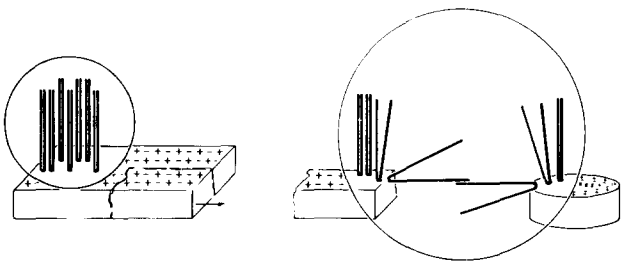

Fig. 6. A concept based on the sample interpretation. The two zones of a bipartite force film (dipole and van der Waals forces, above; ionic forces, below) formed by amphiphilic molecules could respond independently to an energy increment, if the ionic forces were diminished by an electrical milieu. A film change-of-state from solid to fluid, breaking first in the ionic zone, would provide for the formation of interacting lipid domains.

Most of the distinctive shape characteristics had appeared by the third cycle of the film; therefore, the γ–A trace from the third cycle was selected for comparison of shapes. Because shape analysis presents many difficulties for present technology, and because it is a modality in which visual observation serves well, Fig. 3 is included to demonstrate γ–A comparative shapes of different lipids. Certain traces like those of phosphatidylserine and phosphatidylinositol under these conditions (Figs 3b and 3e) fail to give identifiable characteristics.

Fig. 4 demonstrates that the hysteresis loop shape is sensitive to a change in molar ratio. The difference in the shapes of 80% dipalmitoyllecithin:20% phosphatidylinositol (A) from 60% dipalmitoyllecithin:40% phosphatidylinositol (B) is apparent. It should be noted that these shapes are different from both parent films (Fig. 3d and 3e).

DISCUSSION

Changes of shape in the hysteresis loops can be produced by salt concentration, pH, temperature, and water level. For this and other reasons the technique requires further refinements. For this reason also the shapes illustrated are presented, not as definitive, but as an indication of developmental possibilities. An effort was made to minimize experimental variation other than the variation of the molecular species and/or the molar ratios of the film components: film composition was used more or less as an "internal standard". With this technique shape differences based on film composition could be seen.

Interaction of lipid domains was the interpretation desired from the shape differences, because the usual caloric and spectrophotometric criteria were not accessible in this experimental setting. It was anticipated that if Lipid 1 gives a γ–A trace, $y_1 = f(x)$, and Lipid 2 gives $y_2 = g(x)$, then a mixture of these lipids would give $y_{1,2} = \phi[f(x), g(x)]$ in proportion to the molar ratio in the mixture. This appears to be the case with lecithin–cholesterol films.

One is tempted to interpret a trace that is quite different from either of the parent traces as representing interaction at the submolecular, molecular, or domain level (Fig. 4). Such an interpretation might be made on a more certain basis in the case where a unique shape is considered to represent ionic interaction in a film containing charged molecules.

A film composed of 80% lecithin and 20% phosphatidylserine is such a film (Fig. 5). The ionic bonding makes this film perform in a manner different from films like lecithin–cholesterol. As an example, this film has a tensile strength that seems to resist monomolecular collapse but instead appears to fold over on itself. There are characteristics in the trace, moreover, that indicate interaction in the ionic zone of the film.

In Fig. 5 the compression isotherm (lower, progressing from right to left) contains a notch, which can be made to disappear by increasing the subphase salt concentration to 0.016% NaCl. $CaCl_2$ seems to have less effect in this regard than does NaCl. The addition of protein or change of pH can similarly obliterate this notch. Therefore, this notch is interpreted as the effect of ionic forces. When the lipids have been compressed together into a range where the ionic attractive forces become strong enough, the molecules move together more rapidly than the barrier blades are impelling them. The time t and the distance s through which these molecules accelerate could be measured from this type of trace, permitting calculation of the mass and hence size of the then-interacting domains. When the film has undergone maximal contraction due to the ionic forces, the hydrocarbon chains still lack the orientation of the solid phase. Continued compression by the barriers could produce increased parallel ordering of these chains, mesophases, or collapse structures.

Continuing an interpretation of Fig. 5, the first part of the expansion isotherm (upper, proceeding from left to right) shows a rapidly increasing surface tension. The distribution of lipids around the Wilhelmy plate being undetermined, it is possible that the lipids at that stage remain together as a surface lens. The dip in the expansion isotherm, similar to the compression notch, can be altered by changing the pH and salt concentration. This dip, often followed by an increase in surface tension, is interpreted as representing the melting of the ionic zone of the film. It would seem reasonable that the hydrophobic and hydrophilic portions of the film show a differential and sequential response to a given perturbation.

The monomolecular layer of amphiphilic molecules at an aqueous–non-aqueous interface, indeed, could act as a bipartite film with the bonds of the two zones of the film responding to a given energy increment according to the conditions obtaining at the time of the energy incidence. Dipole and van der Waals forces are referred to as "weak forces," whereas ionic forces are called "strong" on the basis of distance between the bodies considered. The equation of Salem[18]

$$W = \frac{A\rho}{4\lambda^2 D^4}\left(3\tan^{-1}\rho + \frac{\rho}{1+\rho^2}\right)$$

indicates that the dispersion energy is proportional to D^{-5}, for a total dispersion energy W, coefficient of dispersion interaction between two basic units A, length of a basic unit λ, the number of basic units in parallel saturated hydrocarbon chains N, chain separation distance D, and $\rho = N/D$. An equivalent expression of the net

potential W between two ions, including the Born and Mayer repulsion potential[19], is:

$$W = \frac{-Q_1Q_2}{D} + b\, e^{-D/a}$$

with charges Q, and constants a and b. Here the energy is proportional to D^{-1}. Therefore, one might expect that there would be a separation distance where ionic and dipole bonding would have equivalent importance in molecular interactions. Inspection of the graphs of the above equations reveals that this distance would be in the neighborhood of 3 Å. Since this is less than the 4.04 Å least-distance between two methyl groups[20], the distance of equal influence would have no meaning for lipid interactions unless the relative strength of the ionic bonding were decreased: without bond-equalizing circumstances expression of the structural and functional significance of the unique lipid compositions in membranes might be difficult. The ionic concentrations, pH, and temperature, carefully controlled in many living systems, could represent important modifies of ionic bonds.

Were the ionic forces to be modulated by such factors so that the expression of dipole and van der Waals forces would become relatively more important, one might expect a monomolecular layer to undergo a change of state in a specific sequence between the two parts of the film. This would depend on which type of bonding, in the present state of the film, would be challenged the most at the moment of arrival of an energy increment or stimulus.

Fig. 6 is a model of a solid bipartite film breaking first in the ionic zone. The hydrocarbon chains are decompressed as a section of film is given more area. Such a section, having a nearly self-stabilizing molecular complement, could undergo rearrangement into a lipid domain. The hydrocarbon chains spreading out from the periphery could interact so as to influence the fluidity characteristics of the film.

Phospholipids in varying compositions are found in all biological membranes. The choice of these ubiquitous building blocks might be based on the sequence of needs in self-assembly. The polar portion of the phospholipid would assist in aggregation, orientation, and slow assembly. Concurrently the closely guarded electrical milieu would modulate the interionic forces of the assembled molecules, thereby stabilizing lyotropic domains and permitting expression of the subtle functions of dipole and van der Waals forces.

ACKNOWLEDGEMENTS

This work was supported in part by grants: HEW 5-S01-RR-5426-09 and American Cancer IN-46J.

Appreciation is expressed to Inocenzio G. Gonzales for technical assistance, to Edwin E. Ely for the use of the Tektronix R 5031 Storage Oscilloscope and C170 Camera System, and to Dr Raymond Adams for analysis of the phospholipid mixture.

REFERENCES

1 Singer, S. J. and Nicolson, G. L. (1972) *Science* 175, 720–731
2 Warren, B. E. (1933) *Phys. Rev.* 44, 969–973
3 Levine, Y. K. and Wilkins, M. H. F. (1971) *Nat. New Biol.* 230, 69–72

4 Steim, J. M., Tourtellotte, M. E., Reinert J. C., McElhaney, R. N. and Rader, R. L. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 104–109
5 Kornberg, R. D. and McConnell, H. M. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 2564–2568
6 McFarland, B. G. and McConnell, H. M. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 1274–1278
7 McConnell, H. M., Wright, K. L. and McFarland, B. G. (1972) *Biochem. Biophys. Res. Commun.* 47, 273–281
8 Frye, L. D. and Edidin, M. (1970) *J. Cell Sci.* 7, 319–335
9 Rouser, G., Yamamoto, A. and Kritchevsky, G. (1971) *Arch. Int. Med.* 127, 1105–1121
10 Leathes, J. B. (1925) *Lancet* 853–856
11 Ladbrooke, B. D., Williams, R. M. and Chapman, D. (1968) *Biochim. Biophys. Acta* 150, 333–340
12 Phillips, M. C., Ladbrooke, B. D. and Chapman, D. (1970) *Biochim. Biophys. Acta* 196, 35–44
13 Joos, P. (1970) *Chem. Phys. Lipids* 4, 162–168
14 Gershfeld, N. L. and Pagano, R. E. (1972) *J. Phys. Chem.* 76, 1244–1249
15 Lusted, D. (1973) *J. Colloid Interface Sci.* 44, in the press
16 Langmuir, I. (1917) *J. Am. Chem. Soc.* 39, 1848–1906
17 Mendenhall, R. M. and Mendelhall, A. L. (1963) *Rev. Sci. Instrum.* 34, 1350–1352
18 Salem, S. L. (1962) *J. Chem. Phys.* 37, 2100–2113
19 Moore, W.J., (1962) *Physical Chemistry*, 3rd edn, pp. 519, Prentice-Hall, Englewood, N.J.
20 Steinfink, H., Post, B. and Fankuchen, I. (1955) *Acta Crystallogr.* 8, 420–424