Hydrolysis of monomolecular layers of synthetic sphingomyelins by sphingomyelinase of *Staphylococcus aureus*

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(Received 16 September 1981/Accepted 11 November 1981)

The enzymic hydrolysis of three synthetic sphingomyelins, spread as monomolecular films at the air/water interface by purified *Staphylococcus aureus* sphingomyelinase was studied. Each of the three sphingomyelins (DL-erythro-*N*-palmitoyl-, -*N*-stearoyl- and -*N*-lignoceryl-sphingosylphosphocholine) has an optimal activity-dependent surface pressure or concentration curve. The optimal surface pressure as well as the optimal surface density for hydrolysis was different for each of the three substrates. This optimum coincides with the liquid-condensed/liquid-expanded phase transition for each of the sphingomyelins. At initial surface pressures (π_0) below the optimum, reaction rates are controlled mainly by surface density of the substrate; above the optimal π_0 , reaction rates decrease with increasing surface pressure. The differences between the three synthetic sphingomyelins are explained by the variation in the degree of asymmetry between their two paraffinic chains.

Rates of enzymic hydrolysis of polar lipid substrates may be related to parameters that characterize the lipid surface, such as surface density of the substrate molecules, their orientation at the interface or the total surface area of the lipid particles (Gatt & Barenholz, 1973; Verger & de Haas, 1976). Studies on the enzymic hydrolysis of SPM in the presence of Triton X-100 (Yedgar & Gatt, 1976) suggested that the density of the substrate molecules at the mixed-micellar surface is a dominant factor affecting the reaction rate. Analysis of the enzymic utilization of 'insoluble swelling' amphiphilic lipid substrates (Small, 1970) in aqueous dispersions is complicated, owing to difficulties in controlling the parameters that affect the interfacial properties of these dispersions (Gatt & Barenholz, 1973). Dispersing these compounds by detergents permits a better definition of the dispersion state, but adds one further component to the already complex system. These difficulties may be obviated when using monomolecular layers. In this form, all the substrate molecules are exposed to the enzyme and the surface characteristics can be controlled and directly studied.

In this paper we studied the enzymic hydrolysis of

Abbreviations used: SPM, sphingomyelin; π , surface pressure; π_0 , initial surface pressure.

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sphingomyelin, spread as a monomolecular layer at an air/water interface by sphingomyelinase of *Staphylococcus aureus*. Three synthetic sphingomyelins having saturated fatty acyl residues of 16, 18 and 24 carbon atoms were used. Bovine brain sphingomyelin was also used to calibrate the measurements with the synthetic sphingomyelins. The rates of enzymic hydrolysis of the lipid in the monomolecular layers were studied as a function of time, surface pressure and surface density.

Materials and methods

Materials

Sphingomyelinase of *Staphylococcus aureus* was prepared by the method of Colley *et al.* (1973). The purified enzyme was stable for at least 2 years when stored in 50% glycerol in 0.05 M-Tris/HCl buffer, pH 7.4, containing 5 mM-CaCl₂ and 2 mM-MgSO₄. Gel electrophoresis carried out with the enzyme preparation showed the presence of two or three protein bands.

D-erythro-Sphingomyelin of bovine brain was prepared, analysed (Shinitzky & Barenholz, 1974) and labelled with ¹⁴C in the methyl group of the choline moiety (Stoffel, 1975). *N*-Palmitoylsphingosylphosphocholine (C₁₆-SPM), *N*-stearoylsphingosylphosphocholine (C₁₈-SPM) and *N*-lignoceryl-sphingosylphosphocholine (C₂₄-SPM), all of DL-

erythro configuration, were gifts from Professor D. Shapiro, Weizmann Institute of Science, Rehovot, Israel; they were further purified by chromatography on silicic acid columns. Their analysis (Barenholz *et al.*, 1976) indicated that the fatty acid residue of each SPM species was more than 99.8% pure and that the long-chain base content of the synthetic compounds was more than 85% of sphingosine, the rest being sphinganine (Barenholz *et al.*, 1976). All lipids were over 99% pure as tested by t.l.c. with a 0.5–1 mg load of each lipid. All solvents were of analytical grade and were used after redistillation.

The concentration of SPM is expressed in mole-equiv. of P (Bartlett, 1959). Protein concentration of the enzyme preparation was determined by the method of Lowry *et al.* (1951).

Measurement of enzymic hydrolysis of sphingomyelin monolayers

Force–area curves were measured by using two systems, one described by Verger *et al.* (1973) and the other described by Quinn & Barenholz (1975). Rates of enzymatic hydrolysis were measured by two parameters; change in surface pressure and surface radioactivity as described by Quinn & Barenholz (1975). All measurements were conducted at $23 \pm 1^\circ\text{C}$.

The highly pure water used in monolayer experiments was obtained by distillation over KMnO_4 immediately before use. Its purity was judged from the interfacial junction potentials, measured using ^{241}Am air-ionizing electrode as described by Quinn & Barenholz (1975). The trough was washed repeatedly with water after each experiment and refilled to the top with the buffer. The surface was cleaned by sweeping several times with a Teflon barrier and aspirating off surface material from the front of the barrier. The buffer in the bulk medium, prepared with the purified water, was 50 mM-Tris/HCl, pH 7.4, containing 5 mM- MgSO_4 (BDH AnalaR). The surface tension of this buffer was the same as that obtained with pure water ($72.6 \text{ mN}\cdot\text{m}^{-1}$ at 23°C).

Monomolecular layers of SPM were spread on the surface by delivering, with the aid of a micropipette, a portion of a solution of the substrate in benzene/chloroform/methanol (5:5:1, by vol.), or in chloroform/methanol (2:1, v/v). The solvent itself had no effect on the surface tension of the buffer.

Two procedures were used for measuring the rate of enzymic hydrolysis. In the first, the substrate film was spread before injection of enzyme into the stirred subphase. In the second procedure, a solution of the enzyme in buffer was poured into the trough and the lipid monolayer was then spread on the surface of the aqueous phase. The effects of the two respective procedures on the reaction rate are

described in the Results section. Reaction rates were measured at constant surface areas by using a 'First Order Trough' (Verger & de Haas, 1976). For more details, see under 'Enzymic hydrolysis of monomolecular layers of SPM'.

Results

Force–area curve of the SPMs

Fig. 1 shows the force–area curves of *N*-palmitoyl-, *N*-stearoyl- and *N*-lignoceryl-sphingosylphosphocoline, all in the *DL*-*erythro* configuration, as well as of bovine brain *D*-*erythro*-SPM. The data in Fig. 1 were obtained by compressing the monolayer with a Teflon barrier using fixed quantities of substrate at the interface. It is noteworthy that the monolayers of the SPMs may be metastable and not at equilibrium (Gershfeld, 1976) since surface-pressure values may be above equilibrium spreading pressure, which is as yet unknown for the SPMs. However, the comparison between the various force–area curves is still valid for the following reasons. (a) All studies were conducted at 23°C , which is below the thermotropic phase transition from gel phase to liquid crystalline phase of the fully hydrated sphingomyelins used in the present study (Barenholz *et al.*, 1976). (b) Stable surface-pressure values were obtained up to $20 \text{ mN}\cdot\text{m}^{-1}$; at higher values the pressure changed with time at a rate of less than $0.2 \text{ mN}\cdot\text{m}^{-1}\cdot\text{min}^{-1}$. (c) The force–area curves were reproducible at different compression

Fig. 1. Force–area curves of monomolecular films at an air/water interface of four SPMs at 23°C . Each curve represents an average of five different experiments.

rates with a maximal variation of less than $1 \text{ mN} \cdot \text{m}^{-1}$ at high pressures. In control experiments where the surface pressure was varied by spreading different quantities of substrate on a constant surface area, identical force–area values were observed. Films obtained by using the latter procedure were stable at all surface pressures used.

Fig. 1 shows that all SPMs exhibited discontinuous force–area curves that displayed transitions from an expanded to a condensed state (Gains, 1966). It is noteworthy that for both C_{24} - and C_{16} -SPM this transition occurred at greater surface pressures than for C_{18} -SPM. Thus below $10 \text{ mN} \cdot \text{m}^{-1}$ the films of C_{16} - and C_{24} -SPM are more expanded than those of C_{18} -SPM.

Fig. 1 also shows that the monomolecular layer formed by sphingomyelin of bovine brain is more expanded than the corresponding films formed from synthetic sphingomyelins at all π values. This might be due to the presence of 30% *N*-nervonyl ($\text{C}_{24:1}$)-sphingosylphosphocholine in this natural SPM (Barenholz *et al.*, 1976). A similar effect was observed when SPM, enriched with nervonic acid was added to an SPM preparation enriched with saturated acids (Rapper *et al.*, 1966). The limiting area per molecule for the bovine brain SPM was about $0.5 \text{ nm}^2/\text{molecule}$. Similar differences were obtained by using natural SPM that had been saturated by hydrogenation (Shah & Schulman, 1967b).

Enzymic hydrolysis of monomolecular layers of SPM

The enzymic hydrolysis of the synthetic SPM monolayers was followed by the reduction of surface pressure. The validity of this method was tested by measuring the rate of hydrolysis of [^{14}C]choline-labelled SPM by the sphingomyelinase of *Staph. aureus*. This was done at two initial surface pressures (π_0), $27 \text{ mN} \cdot \text{m}^{-1}$ and $20 \text{ mN} \cdot \text{m}^{-1}$. Linear rates of hydrolysis (measured as changes in surface pressure or surface radioactivity) were observed at enzyme concentrations from 7 to $34 \mu\text{g}/\text{ml}$. Within this range of protein concentrations the enzyme itself had no effect on the surface pressure.

Ceramide, one product of SPM hydrolysis, is an 'insoluble, non-swelling' amphiphilic lipid (Small, 1970) and therefore remains at the interface, whereas phosphocholine, the second product, diffuses rapidly into the bulk phase (Miller & Ruyschaert, 1971). To confirm that phospho[^{14}C]choline is indeed removed completely from the surface after its release from the SPM substrate, [^{14}C]choline-labelled SPM was spread as a monomolecular layer at the air/buffer interface, using initial surface pressures of 26.5, 21 or $8 \text{ mN} \cdot \text{m}^{-1}$. Enzyme was added to the bulk phase and the enzymic reaction was followed by measuring the decrease in both surface pressure

and surface radioactivity. The bulk phase was then collected and the reaction was terminated by extraction by the procedure of Bligh & Dyer (1959). The radioactivity of the upper aq. methanolic phase was determined. For all π_0 values used, the calculated quantity of phosphocholine recovered in the bulk phase was within 1% of the value obtained by measuring the decrease of the surface radioactivity.

Ceramide has a much lower surface activity than SPM. As a consequence, the increase in mole fraction of ceramide in a mixed ceramide/SPM film is paralleled by reduction in the surface pressure. Fig. 2 shows this as a function of the total number of molecules at the interface. Two curves are shown, one obtained from a monolayer of SPM only, and the other obtained from a mixed film composed of bovine brain ceramide and bovine brain SPM in a 1:1 molar ratio. The total number of molecules in both cases was identical. It is clear that with the latter mixture the surface pressure was about $10 \text{ mN} \cdot \text{m}^{-1}$ lower than that obtained with the equivalent number of SPM molecules.

Fig. 3 shows the time-dependent hydrolysis of monomolecular layers of bovine brain [^{14}C]SPM expressed in terms of surface pressure and radioactivity. This experiment was done at seven different initial pressures, of which only four are shown in the Figure.

The maximal slopes of these sigmoidal curves (i.e., the tangents at the inflection points) were taken as a measure of the reaction rates and related to the initial surface pressure as presented in Table 1. The

Fig. 2. Surface pressure as a function of surface density (molecules per unit area)

Symbols: ●, bovine brain SPM; ○, SPM/ceramide (1:1, v/v). Results are averages of five experiments (for details, see the text).

Table 1. Maximal rates of hydrolysis of bovine brain [^{14}C]SPM at an air/water interface by sphingomyelinase of *Staph. aureus*

The rates were derived from the maximal slopes of Fig. 3. The Table compares the values derived from the curves describing changes of surface radioactivity with time, expressed in molecules hydrolysed/min (dn/dt), with those obtained from the curves describing changes in surface pressure with time ($d\pi/dt$). The first column shows the initial surface pressure π_0 of the films at time zero. The last column gives the ratio between the results in the second and third columns. Results are means $\pm$ s.d. of four to six different experiments.

π_0 ($\text{mN} \cdot \text{m}^{-1}$)	$10^{-15} \times dn/dt$ (molecules $\cdot \text{min}^{-1}$)	$d\pi/dt$ ($\text{mN} \cdot \text{m}^{-1} \cdot \text{min}^{-1}$)	$10^{-15} \times dn/d\pi$ (molecules/ $\text{mN} \cdot \text{m}^{-1}$)
7	6.11 ± 0.3	0.55 ± 0.05	11.1
22	10.50 ± 0.5	0.95 ± 0.06	11.1
32	2.32 ± 0.1	0.23 ± 0.01	10.1

Fig. 3. Hydrolysis of bovine brain SPM, expressed as a decrease of surface pressure (continuous line, roman numerals) and surface radioactivity (serrated line, arabic numerals)

reaction rates were lower at surface pressures of $7 \text{ mN} \cdot \text{m}^{-1}$ and $32 \text{ mN} \cdot \text{m}^{-1}$ than at $22 \text{ mN} \cdot \text{m}^{-1}$, suggesting that maximal enzymic activity is obtained at an optimal surface pressure of about $22 \text{ mN} \cdot \text{m}^{-1}$; above and below this value the reaction rate is lower. Another aspect of Fig. 3 is the irregular shape of the curves describing the time-dependent hydrolysis obtained when π_0 was higher than $22 \text{ mN} \cdot \text{m}^{-1}$ (curves I and II). At high surface pressure the reactions begins with a slow rate, which is accelerated when the surface pressure decreases as the reaction proceeds. The same phenomenon was also observed with each of the three synthetic substrates. The slow phase of these curves is herewith defined as the 'acceleration time' and is calculated by intercepting the tangents of the 'fast' portion of the curve with that of the initial slow portion (see Fig. 3).

Fig. 3 exhibits good agreement between the change in number of SPM molecules in the film (calculated from the change in surface radioactivity (dn/dt) and the change in surface pressure ($d\pi/dt$), with respect to both acceleration time and

Fig. 4. 'Acceleration time' as a function of initial surface pressure (π_0) of monolayers of the four SPMs. For details, see the text.

slope. The reaction rates, derived from dn/dt and $d\pi/dt$ curves, are presented in Table 1.

The good agreement between these two methods is indicated by the almost constant value obtained for $dn/d\pi$ (see Table 1). It is noteworthy that during the SPM hydrolysis its concentration is constantly changing. Therefore, this reaction is not a zero-order one (Verger & de Haas, 1976). This introduced a complication into the kinetic consideration of the rate equations. For the purpose of studying the relation of initial surface pressure to the enzymic activity, we empirically calculate the maximal reaction rates as the tangent to the steepest part of the sigmoidal $\pi-t$ curve (Fig. 3) and relate it to the initial surface pressure. The fact that $d\pi/dt$, from which maximal reaction rates are calculated, as well as dn/dt , remain constant for relatively long periods (see Fig. 3 and Table 1) supports the relevancy of π_0 as an important factor in determining the reaction rate.

Fig. 4 shows the 'acceleration time' as a function of the initial pressure of the film (π_0) for the four substrates. The Figure demonstrates that this parameter varies with the chain length of the fatty acyl

residue of SPM, although for all the four substrate 'acceleration time' becomes obvious only at $\pi_0 >$ optimal π_0 .

Fig. 5 describes the reaction rates for each of the four SPM species as a function of two parameters, the initial surface pressure and the area per molecule of each species. The latter values were derived from the force-area curves of Fig. 1. The data of the experiment of Fig. 5 are summarized in Table 2. They demonstrate that for each of the four respective substrates the maximal rate of hydrolysis was observed at a different optimal pressure. The respective absolute activities observed at the optimal pressure differed with each substrate. It is noteworthy that neither the optimal pressures nor the absolute reaction rates followed the order of increasing chain length of fatty acyl residues on the synthetic SPMs. The optimal initial pressures too seem to coincide with those regions in the curves

where the films change from the expanded to the condensed state (see Table 2).

An experiment was designed to test if the interaction of the enzyme with the SPM molecules in the monolayer was reversible. A monomolecular layer of SPM was spread at π_0 below $22 \text{ mN} \cdot \text{m}^{-1}$, enzyme was added, and when the reaction achieved its fast phase, small unilamellar liposomes of bovine SPM (prepared and fractionated as described by Barenholz *et al.*, 1977) were injected into the subphase. The quantity of liposomes was chosen so that the number of total SPM molecules in the outer layer of these vesicles exceeded those that were in the monolayer by about 10-fold. Addition of these liposomes decreased the rate of hydrolysis of the SPM monolayer, as reflected by $d\pi/dt$, from 1.7 to $0.6 \text{ mN} \cdot \text{m}^{-1} \cdot \text{min}^{-1}$. In a second experiment, liposomes of SPM, whose outer leaflet SPM content exceeded those in the monolayer that were added to

Fig. 5. Rate of enzymic hydrolysis of four SPM monolayers as a function of the respective initial surface pressures (a) and calculated areas per molecule and molecules per unit area (b)

Symbols: ∇ , bovine brain $[^{14}\text{C}]$ SPM; $\circ$, C_{16} -SPM; $\square$, C_{18} -SPM; $\triangle$, C_{24} -SPM. Results are averages of five different experiments.

Table 2. Physical properties of monomolecular films of the four substrates and their related enzymic hydrolysis rates

Substrate	Optimal π_0^a ($\text{mN} \cdot \text{m}^{-1}$)	Transition pressure range ^b ($\text{mN} \cdot \text{m}^{-1}$)	Area/molecule at optimal π_0^b (nm^2)	Relative reaction rate ($d\pi/dt$) at optimal π_0^a ($\text{mN} \cdot \text{m}^{-1} \cdot \text{min}^{-1}$)
Brain SPM	19–22	21–28	1.2	1.0
C_{16} -SPM	16–20	11.5–15.5	0.48	1.49
C_{18} -SPM	7–10	3.8–7.5	0.5	0.68
C_{24} -SPM	12–16	11–15	0.46	0.89

^a Derived from Fig. 5.

^b Derived from Fig. 1.

the subphase before the enzyme by 5- or 10-fold. The presence of the liposomes lowered the rate of hydrolysis of the substrate in the monolayer, as reflected by $d\pi/dt$, from 1.8 (without liposomes) to 1.05 and 0.65 $\text{mN} \cdot \text{m}^{-1} \cdot \text{min}^{-1}$ respectively. Control experiments demonstrated that the SPM in these liposomes was indeed hydrolysed by the enzyme.

Discussion

This paper presents a study of the hydrolysis of monomolecular layers at an air/water interface of four species of sphingomyelin, one from a natural source (bovine brain) and three synthetic compounds (*N*-palmitoyl-, *N*-stearoyl- and *N*-lignoceryl-SPM) by the enzyme sphingomyelinase of *Staph. aureus*.

Hydrolysis of each of the four SPMs at the air/water interface was characterized by biphasic curves, which describe the rate as a function of initial surface pressure or area per molecule (see Fig. 5). Also, at high values of π_0 the time-dependent hydrolysis of each substrate followed a sigmoidal shape and an 'acceleration time' was required to reach the fast reaction rate. The bell-shape curve was previously explained (Shah & Schulman, 1967*a*; Esposito *et al.*, 1973; Verger & de Haas, 1976) by two parameters of the film: (a) the surface concentration of the substrate, expressed as the number of molecules per unit area; (b) the surface pressure of the monomolecular film.

Generally, the above bell-shaped curves may result from a combination of two opposing effects as a consequence of increasing the number of substrate molecules at the air/water interface, as suggested by Shah & Schulman (1967*a*) for the hydrolysis of phosphatidylcholine monolayer by phospholipase A_2 . Although the statistical change for enzyme-substrate interaction increases, the greater surface density might decrease the accessibility of the enzyme to the appropriate site on the substrate molecule. The latter may also be explained by either obtaining a less active conformation of the enzyme or having less enzyme molecules associated with the film. It is rather difficult to distinguish between the two possibilities without measuring the number of active enzyme molecules at the interface.

In our system, irreversible denaturation of the enzyme at high surface pressure can be ruled out, since the rates of hydrolysis increased when the pressure was lowered during the course of the reaction (Fig. 3). The results described in the present paper suggest that a third parameter (i.e., the physical state of the film) is necessary to define a substrate-enzyme interaction in a film. It is clear that one must take into account the observation that the π_0 values resulting in optimal enzymic activity coincide with those pressures where the monolayer

changes state probably from the expanded to the condensed state (Table 2). [The nature of the liquid-expanded/liquid-condensed phase change in insoluble monolayers at the air/water interface is a controversial issue. It was suggested that this phase change is not a true thermodynamic transition and that the liquid condensed state may be metastable (Gershfeld, 1976; Jalal & Zografi, 1979). However, it is possible that if compressional/decompressional time scales are short compared with any long-term transition of film to bulk phase, the use of thermodynamic treatment to this transition is acceptable (Landau *et al.*, 1980).] This suggests that for this system, two distinct regions should be considered in the discontinuous kinetic curves. Below the optimal pressure (i.e., in the expanded state) the dominant fact that determines the enzymic activity is the concentration of substrate at the interface. In this region increasing the pressure does not affect adversely the enzymic reaction. In contrast, in the condensed state, the surface pressure of the monolayer becomes the dominant factor that determines the enzymic reaction.

The transition of monomolecular layer from expanded to condensed state may be analogous to the transition from the gel to liquid-crystalline state in a lipid bilayer (Nagle, 1976; Cadenhead, 1977). But in the monolayer, the phase transition occurs under isothermal conditions and the surface pressure is the variable parameter. This was also shown by pressure-dependent shifts in the phase-transition temperature observed for lipid bilayers (Liu & Kay, 1977) and by changes in the shapes of the force-area curves done with monolayers at various temperatures (Nagle, 1975, 1976; Cadenhead, 1977). In either system the phase transition caused an abrupt change in the free volume and consequently a sharp decrease in the fluidity and the lateral or rotational diffusion of the components of the system.

The results described in the present paper resemble the enzymic hydrolysis of sphingomyelin in a liposomal structure (Cohen & Barenholz, 1978) by the same sphingomyelinase. It is likely that in both systems, distinct domains of gel (condensed) and liquid-crystalline (expanded) phases co-exist (Lee, 1975). A better penetration of the enzyme into the lipid layer possibly occurs in boundary regions between these two distinct phases. Our preliminary studies indicate a marked decrease in the enzymic activity caused by cholesterol present in the monolayer. This is presumably due to suppression of the phase transition by a reduction in the free volume of the liquid-crystalline (expanded) state. Such a 'condensing' effect of cholesterol (Shah, 1970) was also found for films composed of a mixture of C_{24} -SPM and cholesterol (Y. Barenholz & R. Cohen, unpublished work).

The physical properties of an SPM or mixture of SPMs (such as the SPMs isolated from natural sources) are strongly related to the acyl chain composition of the SPM, although for the latter these relations are very complicated (Hertz & Barenholz, 1975; Calhoun & Shipley, 1979; Barenholz & Thompson, 1980). Therefore, we decided to analyse the simpler systems of the three synthetic SPMs.

These (C₁₆-SPM, C₁₈-SPM and C₂₄-SPM) have a simple well defined acyl residue bound in an amide bond to the amino group of the sphingosylphosphocholine. Therefore, differences in parameters that characterize the enzymic hydrolysis of these substrates must be related to the variation in the chain length of the fatty acyl residue. These characteristics indeed varied with the three respective compounds. Thus, maximal reaction rates (Fig. 5) as well as the acceleration times (Fig. 4) were obtained at different initial pressures.

It is obvious that neither surface pressures nor the reaction rates followed the order of increasing chain length of the fatty acyl residues of the synthetic SPMs. As seen in Fig. 5, C₁₈-SPM reaches its condensed state at lower π_0 and yields lower reaction rates, compared with the other substrates. This deviation in the behaviour of C₁₈-SPM may correspond with its unique physical characteristics in a monomolecular film and in a bilayer structure. Its liquid-expanded-to-liquid-condensed transition occurred at lower surface pressure than either C₁₆- or C₂₄-SPM (see Fig. 1). Also, in bilayers, unlike C₁₆- and C₂₄-SPM, which have one gel state, C₁₈-SPM has two different gel-like states [a metastable one, whose transition temperature from gel to liquid-crystalline phase is 45°C, and a stable one, in which the paraffinic chains are more ordered, whose transition temperature is 57°C (Estep *et al.*, 1980)].

In conclusion, the system described in the present paper shows that the surface quality of a substrate, with respect to its interaction with protein, is governed by surface pressure and surface density, but also by the phase behaviour. This is especially true for systems that show discontinuity in their phase behaviour.

We thank Dr. R. Verger for helpful discussions and for the use of his trough and microbalance system. This work was supported in part by NIH grants NS02967 and HL17576.

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