Biochimica et Biophysica Acta, 298 (1973) 769–782

BBA 76250

INTERACTION OF CYTOCHROME *c* WITH LIPID MONOLAYERS

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(Received October 24th, 1972)

SUMMARY

Cytochrome *c* was permitted to react with several lipid monolayers in which surface pressure, lipid charge and unsaturation were varied. Cytochrome *c* interaction with the films caused increased surface pressures, and the magnitude and rate of surface pressure change were compared under a variety of experimental conditions. Large surface pressure changes were associated with more expanded films, whereas greater rates of surface pressure change were associated with favorable charge interaction between cytochrome *c* and the films. Under the most favorable conditions, rates of surface pressure change were limited principally by protein diffusion to the interface. From these data, it is suggested that unsaturation in lipids of biological membranes may help stabilise non-polar protein–lipid interactions, whereas charge interaction may facilitate and direct initial binding of protein to membranes.

INTRODUCTION

Several studies concerned with the interaction of protein with lipid monolayers[1–6] have investigated the manner in which cytochrome *c* complexes with phospholipid. Interest has centred on cytochrome *c* since it is reversibly bound to a specific locus in mitochondria, probably by favorable electrostatic interaction between the positively charged cytochrome *c* and a negative site on the membrane[7]. Green and Tzagaloff[8] proposed that this locus may be rich in cardiolipin, a negatively charged phospholipid, and Quinn and Dawson[2] found that cytochrome *c* does form strong complexes with monolayers of cardiolipin.

The most readily measured parameter of protein–lipid complex formation in monolayers is the change in surface pressure ($\Delta\pi$) which follows protein injection into the subphase. Surface pressure changes under these conditions have been interpreted as resulting from penetration of hydrophobic residues of the protein into the lipid chains[9]. Previous studies[1–6] focused on measurements of $\Delta\pi$ under various conditions. It was found that $\Delta\pi$ is dependent on protein concentration, protein and lipid charge, surface pressure, lipid unsaturation and subphase salt concentration.

Abbreviation: Tricine, tris(hydroxymethyl)methylglycine.

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A second parameter of protein–lipid interactions in monolayers is the rate of surface pressure change, $d\pi/dt$. Quinn and Dawson[1] suggested that the rate of cytochrome *c* penetration into lipid monolayers, measured as $d\pi/dt$, depended upon the relative differences of charge on the protein and lipid. They showed that as phosphatidylethanolamine films became charged with increasing pH of the subphase, the rate of penetration by positively charged cytochrome *c* became maximal.

These results suggested to us that $d\pi/dt$ could give further insight into the mechanism by which proteins interact with lipids. The study reported here is a comparative investigation of $\Delta\pi$ and $d\pi/dt$ as cytochrome *c* interacted with a variety of lipid monolayers. To simplify interpretations, we limited the study to 18 carbon acyl derivatives, and varied surface pressure, charge, and unsaturation of the lipid monolayers.

METHODS

AR (Analytical Reagent) grade NaCl and NaOH (Mallenkrodt), reagent grade HCl (Baker and Adamson), Trizma base (Tris) (Sigma) and Tricine (tris-(hydroxymethyl) methylglycine) (Calbiochem) were used. Stearylamine, stearyl alcohol, and stearic acid (Matheson, Coleman, and Bell) were recrystallized twice from hot ethanol. Oleic acid (Mann Research Laboratories) was used without further purification. Cytochrome *c* (Type VI) was purchased from Sigma.

Ethanol (commercial 95%), AR grade acetone, chloroform, and methanol (Mallenkrodt) were redistilled; hexane (commercial "hexanes") was redistilled over H_2SO_4 (Baker and Adamson). Distilled–deionized water was used.

Stearyl sulfate was prepared by the method of Stirton *et al.*[10] and stearyl phosphate was prepared by the method of Brown *et al.*[11]. Stearyl sulfate and stearyl phosphate were stored in benzene–ethanol (4:1, by vol.); the remaining lipids were stored in hexane.

A circular trough was milled from a solid piece of Teflon to an internal diameter of 7.9 cm and a depth of 2.0 cm; the volume was 100 ml. A barrier was fitted into a slot in the sides of the trough which separated the surface into two compartments. When experiments studying protein–lipid interaction were performed, the lipids were spread onto the larger compartment with an area of 32.6 cm^2. Protein was injected through the surface of the smaller compartment to prevent premature association of protein and lipid. The subphase was stirred by a water-driven magnetic stirrer. The surface tension was measured by the Wilhelmy technique[12] using a platinum plate, 1.00 cm in width, suspended from the balance arm of a recording Cahn RG electrobalance.

The lipids were selected to provide a large variation in lipid surface charge and area per hydrocarbon chain. Charge was controlled either by using lipids with different charged head groups (amine, carboxyl, phosphate, sulfate, or hydroxyl), or by using lipids with a carboxyl group and varying the subphase pH. Surface pressure was controlled by varying lipid surface concentration.

Fig. 1 shows a recorder tracing during a typical experiment. The subphase, used in all experiments, consisted of 0.1 M NaCl and 10 mM buffer. Surface contaminants were removed by repeatedly sweeping the surface with a Teflon bar toward a Pasteur pipette connected to an aspirator. The Wilhelmy plate was flamed

to red heat, cooled briefly in air, and placed on a glass rod extending from the electrobalance. The trough was elevated until the surface of the subphase contacted the Wilhelmy plate (A). The surface tension of the subphase was then recorded. This

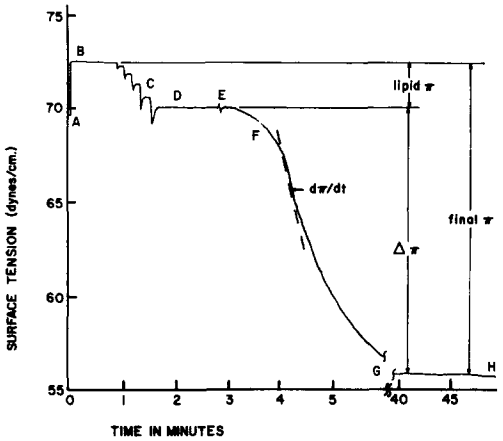

Fig. 1. Recorder tracing of surface pressure during cytochrome *c* interaction with a stearic acid film at lipid $\pi = 2.5$ dynes/cm. Subphase was 0.1 M NaCl, 10 mM Tricine at pH 6.8. Subphase protein concentration after injection (E) was 10 μg/ml. This concentration saturates $\Delta\pi$ under the present conditions.

represented the reference value from which all subsequent readings were substracted to obtain the surface pressure (π). The balance was set to record the range of 55–75 dynes/cm full scale (B) and the lipid was spread from a Hamilton syringe to the desired lipid surface pressure (C). Stirring was initiated (D) and cytochrome *c*, mixed with 0.2 M NaCl to prevent protein flotation, was then injected into the subphase (E). Protein interacting with the monolayer of lipid caused a decrease in surface tension (increase in π) (F). After the maximal rate, $d\pi/dt$, was measured, the recorder was set to a much lower chart speed (G) until no further change in π occurred (H). The distance (B) to (D) measures lipid π (related to lipid surface density), (B) to (H) measures final π (equilibrium π of the protein–lipid complex), and (D) to (H) measures $\Delta\pi$ (change in π due to addition of protein).

RESULTS

Spreading of cytochrome c at the air–water interface

The tendency of cytochrome *c* to spread at the air–water interface in the absence of lipids was initially studied. When cytochrome *c* was injected into the subphase at pH 6.8, the $d\pi/dt$ was 0.37 ± 0.11 dyne/cm per min (three samples). It required several h for the protein to reach a $\Delta\pi$ of 15.5 ± 1.0 dynes/cm. For the purposes of this study, we will assume that interaction between cytochrome *c* and the lipid occurred whenever the $\Delta\pi$ or $d\pi/dt$ values given above for cytochrome *c* alone were affected by the presence of a lipid monolayer.

Fig. 2 shows recorder tracings of π when cytochrome *c* was injected beneath several lipid monolayers. It is obvious that the presence of lipid greatly increased

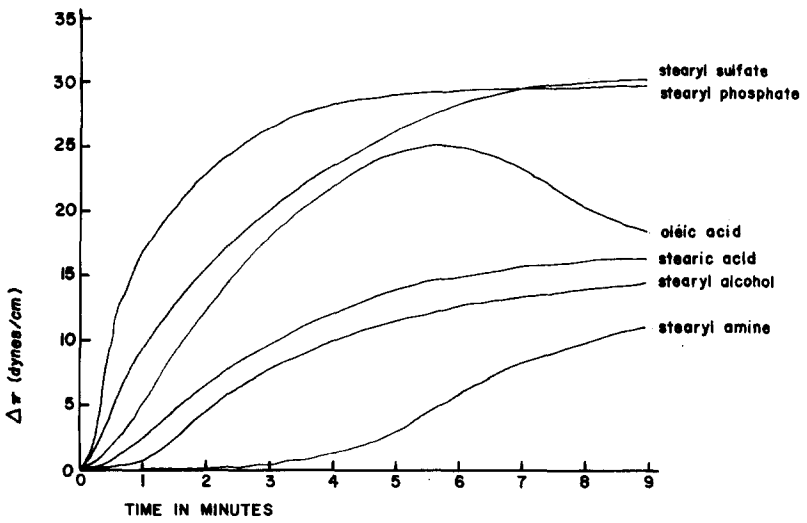

Fig. 2. Recorder tracings showing $\Delta\pi$ for several lipid films following cytochrome *c* injection into the subphase. Lipid π was 6.0 dynes/cm. Conditions as in Fig. 1.

the $d\pi/dt$ values, and had a lesser effect on $\Delta\pi$ values. Table I summarizes some of these results. Two effects were noted: stearylamine, stearyl alcohol and stearic acid did not affect the value of $\Delta\pi$ obtained by spreading cytochrome *c* in the absence of lipid, while the remaining lipids increased $\Delta\pi$ by approximately 10 dynes per cm. However, the presence of any lipid film greatly increased $d\pi/dt$ values. Arranging the lipids by their increasing effect on $d\pi/dt$ gives: stearylamine < stearyl alcohol < stearic acid < stearyl sulfate < stearyl phosphate. This follows the order which is obtained by arranging the lipids according to increasing negative charge, and suggests that $d\pi/dt$ is highly sensitive to charge on the lipid monolayers.

TABLE I

FORMATION OF LIPID–PROTEIN COMPLEXES BY VARIOUS LIPIDS

Subphase was 0.1 M NaCl, 10 mM Tricine, pH 6.8, lipid π was 6 dynes/cm.

Lipid	*Final* π	$\Delta\pi$	$d\pi/dt$
None	15.5	15.5	0.37
Stearylamine	17.5	11.5	3.5
Stearylalcohol	15.5	9.5	6.8
Stearic acid, pH 6.8	17.5	11.5	9.4
Stearyl sulfate	28	22	17.3
Stearyl phosphate	30	24	32.5
Oleic acid, pH 6.8	22.5	16.5	14.5

Effect of lipid π *on* $\Delta\pi$

Cytochrome *c* has been shown to penetrate monolayers of phospholipid[1,2] and it was assumed that $\Delta\pi$ provided a measure of the tendency of the protein to

form complexes by penetration into the lipid film[9]. When lipid π is so great that protein can no longer penetrate ($\Delta\pi=0$), the lipid π at this point has been defined as "limiting π" by Quinn and Dawson[1].

Fig. 3 shows that with stearylamine, stearyl alcohol, and stearic acid, the $\Delta\pi$ of cytochrome *c* decreased over the entire range of lipid π. Limiting π was approximately 17 dynes/cm. Stearyl phosphate, stearyl sulfate, and oleic acid formed a second group with limiting π above 25 dynes/cm. Cytochrome *c* produced $\Delta\pi$ maxima with stearyl sulfate and stearyl phosphate at lipid π values of 2 and 7 dynes/cm, respectively. As lipid π for this group increased beyond 7 dynes/cm, $\Delta\pi$ decreased.

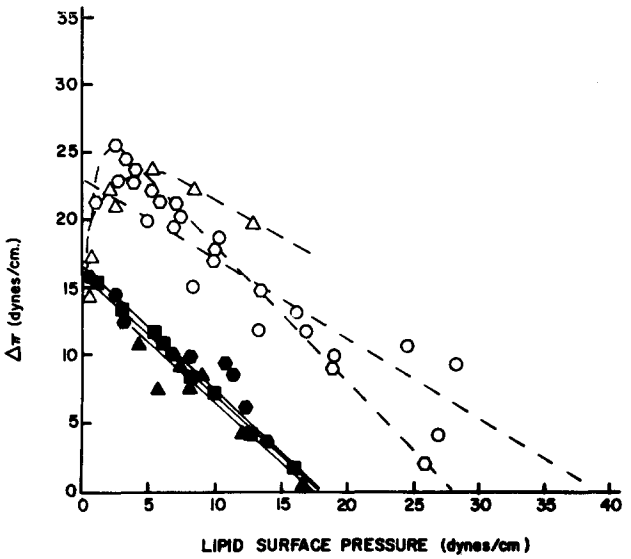

Fig. 3. $\Delta\pi$ for several lipid films as a function of lipid π. Subphase as in Fig. 1. Stearic acid (■); stearylamine (▲); stearyl alcohol (filled hexagons); oleic acid (○); stearyl sulfate (△); stearyl phosphate (open hexagons).

The observed $\Delta\pi$ maxima demonstrate that there are optimum surface lipid concentrations for interaction of cytochrome *c* with each lipid film. Since $\Delta\pi$ for the first group of lipids is essentially the same as $\Delta\pi$ in the absence of lipids, this suggests that in these cases the protein–lipid interactions are relatively weak.

Effect of lipid π on $d\pi/dt$

Fig. 4 shows the effect of lipid π on $d\pi/dt$. Values for $d\pi/dt$ decreased with increasing lipid π for stearic and oleic acid films. However, stearyl phosphate and stearyl sulfate displayed $d\pi/dt$ maxima and relatively high $d\pi/dt$ values. Maximal $d\pi/dt$ occurred at a lipid π of 6 dynes/cm for stearyl phosphate, and at 10–15 dynes/cm for stearyl sulfate. Stearylamine had very low $d\pi/dt$ values and also displayed an apparent $d\pi/dt$ maximum at 5–6 dynes/cm. Stearyl alcohol was unique in that it was the only lipid with which cytochrome *c* would interact at lipid π greater than the limiting π. After protein injection, π rose for several seconds, then slowly decreased to the original lipid π. Thus, $d\pi/dt$ was measurable where no permanent change in π occurred ($\Delta\pi=0$). This may represent an additional type of protein–lipid interaction which is different from that observed with other lipids.

Since the very low $d\pi/dt$ value of cytochrome *c* in the absence of lipid films was increased by the presence of any of the lipids used and decreased with increasing lipid π, all lipid films must have $d\pi/dt$ maxima. For instance, the stearic acid curve in Fig. 4 has a $d\pi/dt$ of 0.37 dynes/cm per min from the data obtained from cytochrome *c* at $\pi = 0$ (absence of lipid). Hence, the maximum occurs somewhere between this value and the first value measured in the presence of lipid at $\pi = 2$ dynes/cm. These maxima, however, are not shown on the graph.

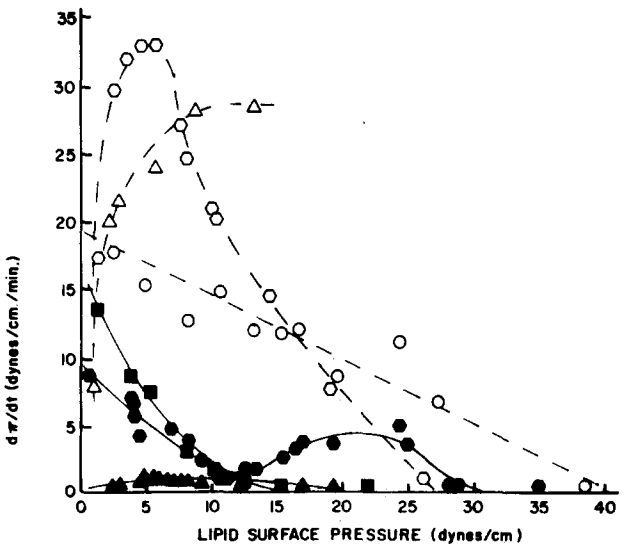

Fig. 4. $d\pi/dt$ for several lipid films as a function of lipid π. Subphase as in Fig. 1. Stearic acid (■); stearylamine (▲); stearyl alcohol (filled hexagons); oleic acid (○); stearyl sulfate (△); stearyl phosphate (open hexagons).

Effect of protein concentration on $d\pi/dt$

Since protein concentration is known to affect values of $\Delta\pi$ (refs 1–3) the effect of cytochrome *c* concentrations on $d\pi/dt$ was determined. With the lipids used in this study, differences in $d\pi/dt$ were most pronounced at a lipid π of 6.0 dynes/cm. Lipid π was therefore adjusted to 6.0 dynes/cm prior to injection of cytochrome *c* into the subphase. The cytochrome *c* subphase concentration ranged from 1 to 20 μg/ml.

Values for $d\pi/dt$ increased with increasing cytochrome *c* concentrations in the subphase (Fig. 5). The order of $d\pi/dt$ values previously measured at a single cytochrome *c* concentration (Fig. 2) was not affected by changing protein concentration by a factor of 20. This result suggests that $d\pi/dt$ values measured in each lipid film are simple functions of protein concentration, and do not depend on more complex events occurring after cytochrome *c* arrives at the interface.

Effect of pH and lipid unsaturation on $\Delta\pi$ and $d\pi/dt$

To test the effect of a single lipid polar group on protein–lipid interactions, lipids containing the carboxyl group were chosen. Since stearic and oleic acid monolayers have different charges on pH 6.8 subphases[13] it was not possible to com-

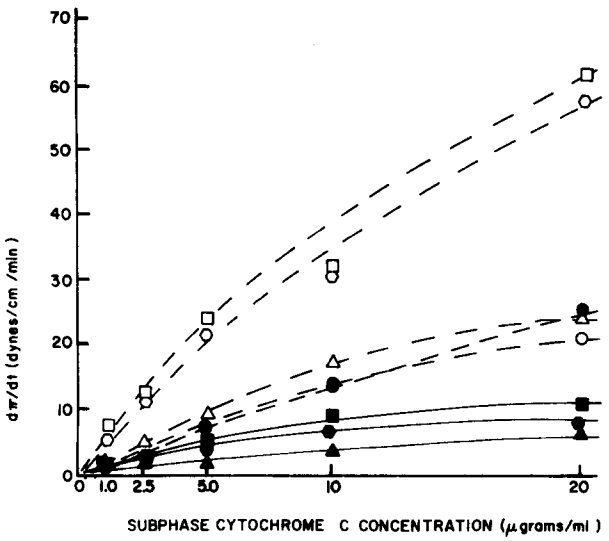

Fig. 5. $d\pi/dt$ as a function of cytochrome *c* concentration in the subphase. Stearic acid, pH 6.8 (■); stearylamine (▲); stearyl alcohol (filled hexagons); oleic acid, pH 5.5 (●); oleic acid, pH 6.8 (○); stearic acid, pH 8.9 (□); stearyl sulfate (△); stearyl phosphate (open hexagons).

pare these lipids at that pH. Consequently, we studied stearic acid at pH 6.8 and 8.9, and oleic acid at pH 5.5 and 6.8. At the lower pH ranges used, both films are essentially uncharged, and at the higher pH ranges, both films have partial negative charges[13]. (More alkaline pH ranges could not be used with oleic acid due to very rapid solubilization of the charged monolayer.) Thus, the effect of unsaturation could be measured when the films were uncharged, and the effect of increasing negative charge on $\Delta\pi$ and $d\pi/dt$ could also be measured in each case. We attempted to use the most narrow possible pH range to minimize charge and pH effects on cytochrome *c*. The net charge on cytochrome *c* changes from +12 to +8 over the pH range of 5.5 to 8.9 (ref. 2).

Fig. 6 shows the relationship between the $\Delta\pi$ of cytochrome *c* and lipid π for stearic and oleic acid. The curves at pH 6.8 were taken from Fig. 2. When cytochrome *c* interacted with stearic acid at pH 8.9, the $\Delta\pi$ was increased by about 5 dynes/cm over that measured at pH 6.8. The opposite effect was noted with oleic acid. However, oleic acid at either pH had greater $\Delta\pi$ values than stearic acid at any lipid π.

When the effect of lipid π on $d\pi/dt$ was studied (Fig. 7), more dramatic effects were seen. The curves at pH 6.8 were taken from Fig. 4. Increasing the charge on stearic acid films caused a large increase in $d\pi/dt$, but did not affect the point where the curve reaches zero. The $d\pi/dt$ maximum observed for stearic acid corresponded to that for stearyl phosphate (Fig. 4). In contrast, the $d\pi/dt$ for oleic acid was not affected by a partial negative charge on the lipid film.

Effect of temperature on $d\pi/dt$

The striking difference between the $d\pi/dt$ for uncharged stearic acid and oleic acid monolayers suggested that the energy required for cytochrome *c* inter-

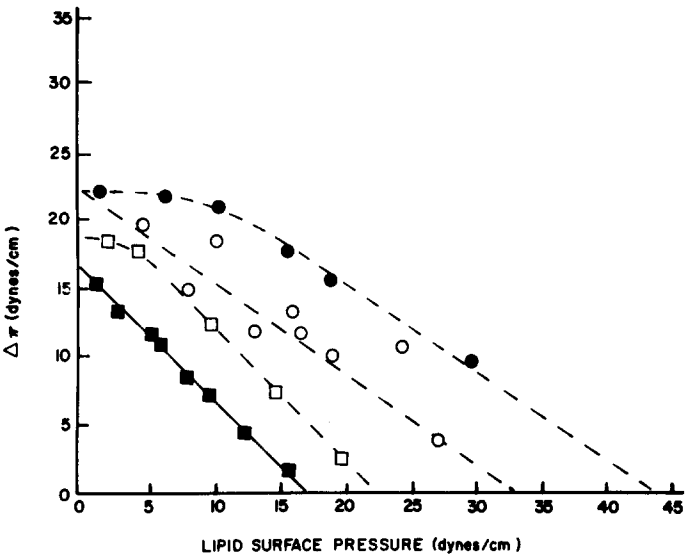

Fig. 6. $\Delta\pi$ as a function of lipid π for stearic acid and oleic acid films at different pH ranges. Cytochrome *c* subphase concentration was 10 μg/ml. Stearic acid, pH 6.8 (■) pH 8.9 (□); oleic acid pH 5.5 (●) pH 6.8 (○).

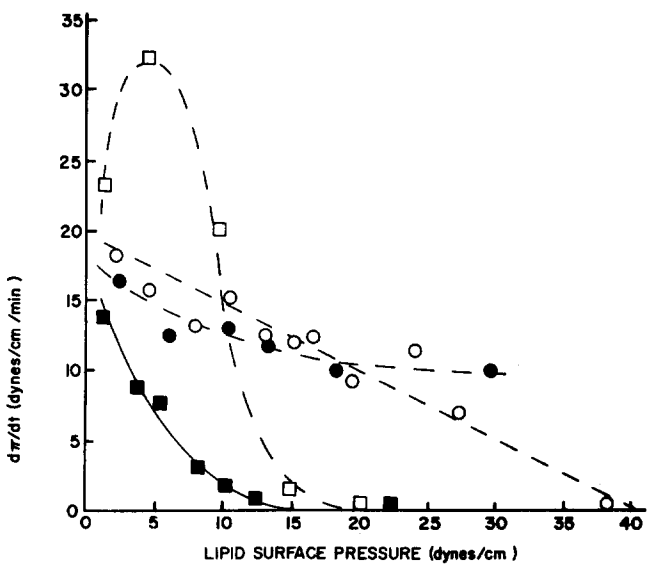

Fig. 7. $d\pi/dt$ as a function of lipid π for stearic acid and oleic acid films at different pH ranges. Cytochrome *c* subphase concentration was 10 μg/ml. Stearic acid, pH 6.8 (■) pH 8.9 (□); oleic acid, pH 5.5 (●) pH 6.8 (○).

action with these lipids was quite different. We therefore studied the temperature dependence of this reaction by measuring $d\pi/dt$ between 18 and 42.5 °C. The subphase was kept at pH 5.5, where both lipid films are nearly uncharged, to test only the effect of unsaturation. Lipid π was held at 6.0 ± 0.5 dynes/cm.

These results are shown in Fig. 8. Using the equation of Eley and Hedge[14]:

$$\frac{dn_{ads}}{dt} = \frac{n_{ads}}{\Delta\pi} \cdot \frac{d\pi}{dt} \cdot \text{molecules/s}$$

where n_{ads} is the number of adsorbed molecules, and plotting log $d\pi/dt$ *vs* $1/T$, the activation energy was calculated to be 11.5 kcal/mole for stearic acid and 4.8 kcal/mole for oleic acid.

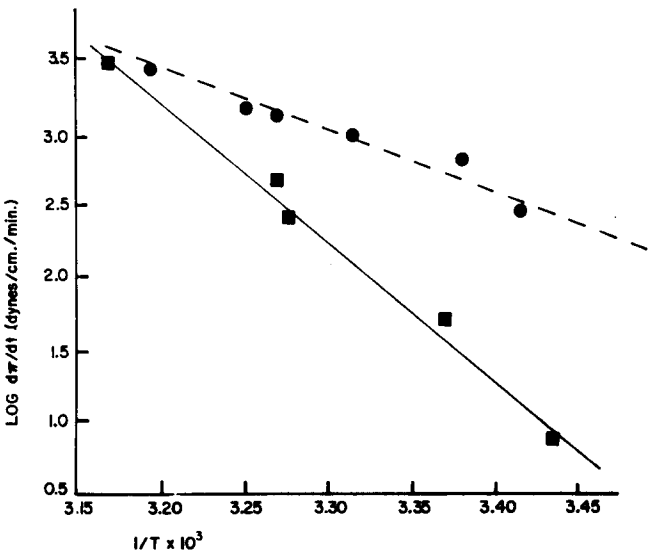

Fig. 8. Arrhenius plot of stearic acid and oleic acid films. Subphase was 0.1 M NaCl, 10 mM Tricine, pH 5.5. $d\pi/dt$ was measured following cytochrome *c* injection to a subphase concentration of 10 μg/ml. Stearic acid (■), oleic acid (●).

DISCUSSION

The rationale of the present study was to attempt to model protein–lipid interactions by using monolayer systems. Certainly it is clear that some form of interaction occurs between protein and lipid in this model, but it is difficult to be precise in defining extent and strength of the interaction in terms of $\Delta\pi$ and $d\pi/dt$. One may imagine that some portion of the cytochrome *c* molecule penetrates the lipid monolayer, and that as more cytochrome *c* interacts in this fashion, surface pressure increases. In this interpretation, $\Delta\pi$ depends on interaction of protein with the non-polar portion of the lipid film, and conditions which affect the non-polar phase would be expected to affect $\Delta\pi$ values. In contrast, values of $d\pi/dt$ do not necessarily reflect the strength of the interaction, but more likely depend on the rate of arrival of cytochrome *c* at the interface and the rate at which energy barriers to penetration are overcome. We will generally follow the above interpretations of $\Delta\pi$ and $d\pi/dt$ in discussing the results, but the uncertainty of such interpretations are a distinct limitation on the present use of monolayers as model systems for protein–lipid interactions.

This investigation has established two relationships between lipid film pro-

perties and protein–lipid interaction in monolayers: (1) The extent of interaction, measured by $\Delta\pi$, is principally controlled by the expanded or condensed character of the lipid film. More expanded films generally produced greater $\Delta\pi$ values when interacted with cytochrome *c*. The expanded nature of the film may be controlled either by surface pressure, by surface charge, or by unsaturation of the hydrocarbon lipid chains. (2) The rate of interaction, measured by $d\pi/dt$, is chiefly determined by favorable electrostatic interactions between the cytochrome *c* and the lipid. Thus, cytochrome *c* had much greater rates of interaction with negatively charged films than with neutral or positively charged films.

$\Delta\pi$ and $d\pi/dt$ as measures of protein–lipid complex formation

In experiments with the variety of lipid films described previously, we found that the dominant factor controlling $\Delta\pi$ could most generally be attributed to the expanded character of the film. Larger $\Delta\pi$ values were associated with conditions which produced more expanded films, such as lower lipid π and unsaturation. Negative charge on the monolayer had a dual effect, in that it seemed to increase $\Delta\pi$ both by producing expanded monolayers as in stearyl sulfate and stearyl phosphate, and by permitting favorable charge interaction between cytochrome *c* and the films.

One test of this generalization was to use stearic and oleic acid films, in which the head groups are equivalent. The charge on the films could be varied by controlling the pH of the subphase, and the expanded character of the film was a function of whether or not a 9–10 *cis* double bond was present. Thus, at lower pH ranges and a lipid π or 6 dynes/cm, both films are essentially uncharged but oleic acid films have a more expanded character. As pH is increased, both films take on increasing negative charge, and any additional effect of charge on $\Delta\pi$ values would be determined.

In general, the films behaved as expected in this test. Uncharged stearic acid films had lower $\Delta\pi$ values than oleic acid films at similar lipid π. Furthermore, as higher pH produced increasing negative charge on the stearic acid films, the values of $\Delta\pi$ increased but did not exceed those of oleic acid at similar lipid π. Unfortunately, the experiment was limited by the solubility of oleic acid films, since oleic acid rapidly disappeared from the interface at higher pH (ref. 13). This probably accounts for the decreased $\Delta\pi$ values of oleic acid films at pH 6.8, a result not in agreement with the generalization that negative charge also exerts an effect in determining the extent of protein–lipid interaction.

The values obtained for $d\pi/dt$ in this experiment were also limited by the solubility of oleic acid. As pH was increased and the films became partially negatively charged, $d\pi/dt$ of stearic acid films greatly increased and exceeded those of oleic acid films. This followed the generalization that charge is dominant in controlling $d\pi/dt$ values. However, values of $d\pi/dt$ for oleic acid films did not increase with increasing pH. This again was probably due to a loss of oleic acid from the interface during measurements, with the result that $d\pi/dt$ values were lowered.

We would again emphasize that although charge seems dominant in controlling $d\pi/dt$, the expanded character of the film also plays a role. For instance, when oleic and stearic acid films were compared at low pH ranges where both are uncharged (Fig. 7) $d\pi/dt$ was greater for oleic acid. This might be expected, since

presumably energy barriers to penetration by cytochrome *c* would be lower in relatively expanded films. In fact, activation energies calculated from Arrhenius plots (Fig. 8) were significantly lower in oleic acid films (4.8 kcal/mole) compared with stearic acid films (11.5 kcal/mole). This suggests that the condition of the lipid chains also has an effect in determining how readily cytochrome *c* may penetrate lipid films.

The role of protein diffusion in controlling $d\pi/dt$

It is clear that $d\pi/dt$ values are basically a function of the rate at which protein molecules arrive at the interface and the rate at which energy barriers to penetration are overcome. Certainly diffusion rates limit the maximum rate at which cytochrome *c* arrives at the interface. It is therefore interesting to calculate if the highest $d\pi/dt$ values can be predicted from a simple consideration of the rate of protein diffusion to the interface. The following eqn[15]:

$$dn/dt = \frac{(D/\pi)^{\frac{1}{2}}}{1000} \cdot ct^{-\frac{1}{2}}N \tag{1}$$

gives the rate of arrival of the protein to a surface without stirring, where *c* is the bulk concentration of the protein in moles/l; *n*, number of adsorbed molecules per cm^2 of surface after time *t* (in s); *D*, diffusion coefficient of the protein ($1.3 \cdot 10^{-6}$ cm^2/s for cytochrome *c*, (ref. 16)); *N*, Avogadro's number; and $\pi = 3.1416...$ This integrates to:

$$n = 2\frac{(D/\pi)^{\frac{1}{2}}}{1000} \cdot ct^{\frac{1}{2}}N \tag{2}$$

This relationship was used by Steinemann and Läuger[4] to calculate diffusion of cytochrome *c* to a lipid–water interface. The equation is only accurate to a factor of 3 or 4 (ref. 15) and does not account for stirring.

When the subphase is stirred either mechanically or by convection[15]:

$$dn/dt = \frac{(D/\delta x)}{1000} \cdot cN \tag{3}$$

where δx is the depth of the unstirred layer. To correct for the filling of the surface by protein:

$$dn/dt = \frac{(DN/\delta x)}{1000} \cdot c(1 - \theta) \tag{4}$$

where θ = fraction of the surface already covered. Using Eqn 4 to calculate dn/dt at $\theta = 0$, assuming $\delta x = 0.001$ cm (ref. 17), and $c = 10$ μg/ml (0.8 μM):

$$dn/dt = 6.2 \cdot 10^{11} \text{ molecules/s per cm}^2$$

This is the maximal rate at which cytochrome can arrive at the interface assuming instantaneous mixing of the protein.

Assuming hexagonal packing of the cytochrome *c* at the interface, or $1.2 \cdot 10^{13}$ molecules/cm^2 (ref. 4), t_{max}, the time required to form a monolayer of cytochrome *c* at the maximal rate, is given by:

$$t_{max} = (1.2 \cdot 10^{13} \text{ molecules/cm}^2)/(6.2 \cdot 10^{11} \text{ molecules/s per cm}^2)$$
$$= 19 \text{ s}$$

It must be emphasized that this number is obtained under the assumption that dn/dt, the number of cytochrome *c* molecules arriving at the interface per unit time, remains constant at its maximum value until the cytochrome *c* forms a monolayer and reaches its equilibrium value ($\Delta\pi$).

If we assume that at equilibrium, at least a monolayer of cytochrome *c* has interacted with the lipid monolayer[1–3] and that dn/dt is maximal at $t=0$ (actually, dn/dt is somewhat slower due to the time required for complete mixing) then we can calculate maximal values of $d\pi/dt$ for each lipid. The $\Delta\pi$ measured for any lipid is taken to be the value in dynes/cm produced by the reaction of a monolayer of cytochrome *c* with the lipid film. Thus, in the case of stearyl alcohol, $\Delta\pi$ was 17.5 dynes/cm and diffusion would produce a monolayer of cytochrome *c* at the interface in 19 s, as calculated above. The maximal value for $d\pi/dt$ would then be

$$\frac{17.5 \text{ dynes/cm}}{19 \text{ s}} \cdot 60 \text{ s/min} = 53 \text{ dynes/cm per min}$$

These calculations are summarized for all lipid films in Table II. It is apparent that the presence of any lipid monolayer, even if unfavorably charged, greatly increased the rate at which cytochrome *c* interacts with the interface to produce a measurable change in surface pressure. This may be considered in terms of the number of cytochrome *c* molecules which must approach the interface before one penetrates. For instance, in the absence of a lipid monolayer, approximately 130 molecules of cytochrome *c* must approach the interface for each one which penetrates, whereas if a positively charged stearylamine film is present, this value is decreased to 36. Under the most favorable circumstances with negatively charged films, only 2–3 molecules approach the film for each which penetrates.

TABLE II

COMPARISON OF CALCULATED AND OBSERVED $d\pi/dt$

The ratio of calculated to observed $d\pi/dt$ values estimates the number of cytochrome *c* molecules approaching the surface before one molecule actually penetrates.

Lipid	*π (dynes/cm) at measured $d\pi/dt$ maxima*			*Maximum $d\pi/dt$ (dynes·cm·min)*		*$d\pi/dt$ calculated observed*
	Final π	*Initial π*	*$\Delta\pi$*	*Observed*	*Calculated*	
None	15.5	0	15.5	0.37	48	130.0
Stearylamine	17.5	5	12.5	1.5	39	36
Stearyl alcohol	17.5	<1	17.0	10.0	53	5.3
Stearyl sulfate	32.5	10	22.5	27.5	70	2.5
Stearyl phosphate	27.5	5	22.5	33.5	70	2.0
Stearic acid, pH 6.8	17.5	<1	17.0	15.0	53	3.5
Stearic acid, pH 8.7	22.0	5	17.0	32.5	53	1.6
Oleic acid, pH 5.5	22.5	<1	22.0	18.0	68	3.8
Oleic acid, pH 6.8	22.5	<1	22.0	20.0	68	3.4

One assumption which is not well controlled in the above analysis is the value for δx, the depth of the unstirred layer. For the purposes of the calculation, δx was assumed to be 10 μm (ref. 17) but if it were larger, the calculated $d\pi/dt$ values would be proportionally smaller and closer to the observed values. A second assumption is that the same conformational change of cytochrome *c* at the interface is responsible for $\Delta\pi$ under all conditions. This is a reasonable assumption for $\Delta\pi$ produced by cytochrome *c* penetration of the nonpolar phase of the lipid films, since all were 18 carbon acyl derivatives, but probably is not true for $\Delta\pi$ measured in the absence of lipid films. In the presence of a lipid film the interface is in fact nearly filled with lipid molecules. Surface pressure changes may therefore be produced by only a small segment of each cytochrome *c* molecule penetrating the nonpolar lipid phase. However, in the absence of a lipid film the interface is obviously empty prior to injection of cytochrome *c*, and the protein must fill the interface itself to produce surface pressure changes. It is probable that much more extensive unraveling of cytochrome *c* is required to produce $\Delta\pi$ values equivalent to those seen in the presence of lipid films. These more extensive conformational changes probably explain the great difference in $d\pi/dt$ values measured in the absence and presence of lipid films.

Relation to biological systems

Application of this understanding to biological systems is limited by the complex composition of membranes. It is difficult even to apply the model to mechanisms of cytochrome *c* binding to mitochondrial membranes, since there is no evidence that cytochrome *c* is capable of hydrophobic penetration reactions with inner mitochondrial membrane sites. Finally, the present results are derived from monolayers, and the extent of protein penetration in other model membrane systems is unknown. Some evidence suggesting penetration reactions of protein with lipid bilayers was provided by Kimelberg and Papahadjopoulos[18] who found that proteins which penetrated monolayers also increased the permeability of sodium through phospholipid vesicles. This could be understood if protein were penetrating the lipid bilayer and producing polar pores through which sodium could pass.

With all its limitations, however, there is no doubt that protein–lipid interactions in monolayers are examples of potential reactions which may occur in the cell. One may speculate that certain newly synthesized proteins bind to membranes by penetration reactions analogous to those which occur in monolayers. Our results suggest that unsaturation in the lipid moiety could stabilize such complexes, and that favorable electrostatic interactions between the protein and specific lipids in the membrane could enhance binding rates and provide specificity to the area of the membrane in which the binding reaction took place.

ACKNOWLEDGEMENTS

This work was supported in part by N.S.F. Grant GB 23187.

P.D.M. gratefully acknowledges assistance by Kiyo Morse in preparing the manuscript.

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