The Penetration of Serum Albumin into Phospholipid Monolayers of Different Fatty Acid Chain Length and Interfacial Charge

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1. The highest surface pressure of phosphatidylcholine monolayers allowing penetration of delipidated serum albumin decreased in the order dibehenoyl > distearoyl > dipalmitoyl = dimyristoyl. This pressure was not related to the area occupied or to the space available between the phospholipid molecules at the interface. 2. Penetration of albumin into yeast phosphatidylcholine monolayers was increased by adding a small percentage of long-chain anions (phosphatidic acid, diacetylphosphoric acid) to the film but only when the protein was below its isoelectric point (i.e., positively charged). 3. Stearylamine added to phosphatidylcholine monolayers had no effect on albumin penetration even when the protein was oppositely charged to that of the phospholipid/water interface. 4. The results are discussed in relation to the activation of certain phospholipases by anionic amphipathic substances.

We have previously studied some of the factors that control the penetration of proteins into monolayers of lipids orientated at the air/water interface (Quinn & Dawson, 1969a,b, 1970). The upper film-pressure limit at which penetration of cytochrome c occurred was not influenced by hydrogenation of the hen's-egg phosphatidylcholine or phosphatidylethanolamine used to form a monolayer although it was decreased somewhat by using a (dipalmitoyl) phosphatidylcholine monolayer. This suggested that the interaction might be more influenced by the length of the phosphatidylcholine fatty acid chains than by their degree of unsaturation. The penetration of cytochrome c was markedly increased by mixing a small percentage of an anionic lipid (cardiolipin) with the phosphatidylcholine (Quinn & Dawson, 1969a,b). Further, the penetration of this protein into phosphatidic acid and cardiolipin monolayers was considerably decreased in the presence of Na-sodium chloride and it was concluded that ionic forces facilitated the interaction.

The present studies were undertaken to examine some additional parameters influencing the penetration of protein into phospholipid monolayers. The effect of increasing the fatty acyl chain length of the phospholipid used to form the monolayer on the penetration of serum albumin was observed. Further, changing the interfacial charge of the monolayer by adding appropriate cationic or anionic amphipaths or adjusting the subphase pH to values above and below the isoelectric point of the protein has enabled us to study the importance of the electrostatic status of the components of the interaction. It was hoped that the principles involved in such interactions might act as a suitable model to explain the interaction of certain phospholipases on their substrates. Although a direct method would have been preferable this is impracticable because apart from the difficulties of obtaining phospholipases of sufficient homogeneity and stability, rapid changes in the composition of the substrate monolayer when hydrolysis commences mask the effect of the preliminary phospholipid-protein interaction.

EXPERIMENTAL

Details of the experimental techniques used for spreading monolayers and measuring the surface pressure were as described by Quinn & Dawson (1969a). Individual films were spread for each experiment and the pressure was adjusted to the required value by compression. The protein was injected into the magnetically stirred subphase at room temperature (18°C) and the pressure increment (Δσ) was continuously recorded until equilibrium had been reached. Samples of dimyristoyl-, dipalmitoyl-, distearoyl- and dibehenoyl-sn-glycero-phosphorylcholine were generously provided by Dr M. C. Phillips (Unilever Research Laboratories, Welwyn Garden City, Herts., U.K.). Yeast phosphatidylcholine was prepared as described by Quarles & Dawson (1969) and phosphatidic acid by the method of Dawson &
Hemington (1967). Didetylphosphoric acid was obtained from Albright and Wilson Ltd., London W.1, U.K. The purity of all phospholipids was checked by t.l.c. Crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) was delipidated by the method of Chen (1967); this was found not to change the optical-rotatory-dispersion spectrum significantly.

RESULTS

Fig. 1 shows the total surface pressure increase ($\Delta\pi$) that occurred when delipidated serum albumin was added to the subphase below monolayers of phosphatidylcholines with fatty acyl chains of 14, 16, 18 and 22 carbon atoms respectively. With the two shorter-chain phosphatidylcholines an increase in the initial starting pressure of the monolayer caused a proportional decrease in the pressure increment when a constant amount of serum albumin was added to the subphase. Similar relationships have been observed on the interaction of cytochrome $c$ with phospholipid monolayers (Quinn & Dawson, 1969a,b), $\gamma$-globulin and dihydroceramide lactoside monolayers (Colacicco, Rapport & Shapiro, 1967) and the apoprotein of high density plasma lipoprotein with phosphatidylcholine (Camejo, Colacicco & Rapport, 1968).

The highest initial pressure of the film that allowed penetration was about 20 dyn/cm for both the (dimyristoyl) and (dipalmityl) phosphatidylcholine monolayers. This cut-off point was increased to 35 and 41 dyn/cm for (distearoyl) and (dibehenoyl) phosphatidylcholine respectively. Moreover, both the $\Delta\pi$–initial pressure curves for the longer-chain phosphatidylcholines showed a change in the constant of proportionality at about 17 dyn/cm which was not observed with (dimyrystoyl) and (dipalmityl) phosphatidylcholines.

The penetration of serum albumin into monolayers of yeast phosphatidylcholine containing predominantly palmitoleic acid and oleic acid (Shah & Schulman, 1965) shows a cut-off point at 21 dyn/cm (Fig. 2a). There appeared to be no significant difference in the $\Delta\pi$–initial pressure relationship when the subphase was buffered to pH 4.5 or pH 5.5. Since serum albumin has an isoelectric point at about pH 5 (Quinn & Dawson, 1969a) this would mean that the globular protein in solution would possess a small net positive or negative charge in the two instances.

The introduction of stearylamine into the phosphatidylcholine monolayer (1:9 molar ratio) produced no significant increase in the penetration of the albumin (Fig. 2b) with the cut-off point if anything slightly lower than that for the pure phosphatidylcholine film both at subphase pH values of 4.5 and 5.5. Even with a subphase at pH 7.5, which would result in an appreciable net negative charge on the protein (Quinn & Dawson, 1969a), the penetration into the positively charged film was only marginally increased.

In contrast, adding phosphatic acid (1:9 molar ratio) to introduce surplus negative charges on the interface produced a marked increase in the ability of albumin to penetrate the film at higher pressures, providing that the protein possessed a net positive charge (i.e. subphase at pH 4.5) (Fig. 2c). With a net negative charge on the albumin (i.e. subphase at pH 5.5) the penetration into the mixed phosphatidylcholine–phosphatic acid film was about the same as into a pure phosphatidylcholine film. (Fig. 2a). The introduction of 10% dietylphosphoric acid into the monolayer likewise resulted in an enhanced penetration of albumin providing the subphase pH was 4.5 (Fig. 2d). The addition of 5mM-Ca$^{2+}$ resulted in a somewhat decreased penetration at higher pressures, as did adjusting the pH to 5.5. Both these manoeuvres decreased the penetration until it was no greater than with a pure phosphatidylcholine monolayer.

DISCUSSION

The increase in surface pressure achieved on adding protein below a monolayer of lipid is generally accepted as resulting from the physical penetration of at least part of the protein molecule between the lipid molecules orientated at the air/water interface. However, it would perhaps be unwise to assume that there is necessarily a simple proportionality between the 'volume' of protein penetrating into the film and the surface pressure.

Fig. 1. Pressure increments ($\Delta\pi$) on adding serum albumin below monolayers of phosphatidylcholine of various fatty acid compositions. Protein (200 $\mu$g) was added to a subphase of 10mM-sodium acetate–acetic acid buffer, pH 4.5. Fatty acid compositions were: $\bigcirc$, dibehenoyl; $\bullet$, distearoyl; $\bigtriangleup$, dimyristoyl; $\Delta$, dipalmityl.
Fig. 2. Pressure increments ($\Delta \pi$) on adding serum albumin below monolayers of yeast phosphatidylcholine alone or mixed with amphipathic substances. O, Subphase 10 mM-sodium acetate–acetic acid buffer, pH 4.5; $\Delta$, subphase 10 mM-sodium acetate–acetic acid buffer, pH 5.5; $\Box$, subphase $5\text{mM-Na}_2\text{HPO}_4$–$\text{NaH}_2\text{PO}_4$ buffer, pH 7.5. $\bullet$, $\triangle$, with $5\text{mM-CaCl}_2$ added to the subphase. (a) Phosphatidylcholine; (b) phosphatidylcholine+stearylamine (9:1 M); (c) phosphatidylcholine+phosphatidic acid (9:1 M); (d) phosphatidylcholine+dicetylphosphoric acid (9:1 M).

increment. The latter would presumably be related to the ‘volume’ by the factors that would include the form of the monolayer’s force/area curve. Moreover, it is clear that the penetration need not necessarily extend into the hydrophobic regions of the lipid monolayer. Thus short-chain aliphatic amines added below monolayers of phosphatidylinositol can cause an appreciable pressure increment even though their carbon chains are unlikely to reach the fatty acid region of the film (Hauser & Dawson, 1968).

We have shown (Quinn & Dawson, 1970) that when serum albumin is added below very low pressure phospholipid monolayers (2 dyn/cm), there is initially a penetration of whole protein molecules between the lipid molecules of the film, which then completely unfold at the phase interface. As the pressure increases the molecules begin to adsorb in a different way with more limited penetration either by the side chains of amino acids or by unfolded regions of the peptide chain. However, there is no evidence of this change in the manner of penetration in the present experiments. The $\Delta \pi$–initial film pressure curves show no discontinuity at a film pressure increase of 8 dyn/cm, which we have observed to represent the critical pressure (serum albumin into phospholipid monolayers) for the two types of penetration (Quinn & Dawson, 1970).

The phosphatidylcholines of various fatty acyl chain length used in the present studies show considerable differences in their force–area per molecule curves (Phillips & Chapman, 1968), (Dibehenoyl) and (distearoyl) phosphatidylcholine.
monolayers are condensed at room temperatures whereas (dimyristoyl) phosphatidylcholine monolayers are liquid-expanded. This means that the pressure limit of penetration cannot be correlated with the size of the gaps between the liquid molecules at the interface since (dimyristoyl) phosphatidylcholine, which has the most space available at a given pressure, penetrates less readily than the (distearoyl) and (dibehenoyl) phosphatidylcholine monolayers. Further, there is no discontinuity in the $\Delta\pi$-initial pressure curve for (dipalmitoyl) phosphatidylcholine where a transition occurs from the liquid-expanded to the condensed state as the surface pressure is increased above 10 dyn/cm under the prevailing temperatures in our system (Phillips & Chapman, 1968). Large differences in the area occupied by phosphatidylcholine molecules brought about by hydrogenation of the fatty acid chain appear to cause little change in the upper surface-pressure limit of cytochrome c penetration (Quinn & Dawson, 1969a, b). The marked increase in the ability of albumin to penetrate the longer-chain phosphatidylcholine films at higher pressures may mean that whole hydrophobic regions of the peptide chains are unfolding to penetrate between the fatty acid chains of the lipid film. It has been pointed out by Haydon & Taylor (1963) that the hydrophobic side chains of amino acids in the peptide chain would not be long enough to penetrate to the hydrophobic region of a phospholipid monolayer without some degree of unfolding. Above a certain fatty acid concentration the binding of hen’s-egg albumin to short-chain fatty acids in the bulk phase increased as the chain length of the fatty acid was extended (Bull & Breese, 1967). However, Spector, John & Fletcher (1969) found that delipidated serum albumin bound palmitic acid more tightly than stearic acid. This may represent rather a specific type of reaction since serum albumin is known to be a carrier of free fatty acids in the plasma and to possess selective binding sites for these acids. Eley & Miller (1960) found that the degree of penetration of bovine serum albumin into fatty acid monolayers increased in the sequence myristic acid, palmitic acid, stearic acid. In these experiments however, the specific binding sites for fatty acids were probably already filled since the albumin was not delipidated. Colacicco et al. (1967) have reported that the penetration of $\gamma$-globulin into monolayers of dihydroceramide lactoside at an initial pressure of 2 dyn/cm increased with increasing chain length at 35°C but at 25°C dihydroceramide C$_{18}$ derivative interacted with the protein less than did the dihydroceramide C$_{14}$ derivative. The penetration of serum albumin into yeast phosphatidylinositol monolayers was not appreciably influenced by the sign of the net charge on the protein molecule or by adding Ca$^{2+}$ to the subphase. This is consistent with the zwitterionic nature of the lipid in the pH range studied (Dawson 1968), which would result in a minimal influence of ionic forces on the penetration. Again, although yeast phosphatidylcholine produces a highly expanded monolayer (Shah & Schulman, 1965) due to its high monoene fatty acid content, the maximum pressure that allowed penetration was no greater than for the saturated phosphatidylinositol of equivalent chain length (cf. Figs. 1 and 2a). It would appear that the limiting pressure of penetration depends more on the work necessary for penetration to occur rather than on the theoretical space available between the phospholipid molecules.

The introduction of the anionic phospholipids (phosphatidic acid or dicetylphosphoric acid) is unlikely to affect appreciably the space available between the lipid molecules, and, providing the protein is positively charged, signs of an electrostatically facilitated penetration are evident. Thus the penetration is decreased if the protein is made negatively charged by changing the subphase pH or by adding Ca$^{2+}$ to the subphase to counter the negative charge on the lipid interface. The pH changes involved in changing the net charge on the protein would not be expected to cause any significant changes in the ionization of the film. At pH 4.5-5.5 both phosphatidic acid and dicetylphosphoric acid have one ionized anionic site; the second site on phosphatidic acid is not likely to be ionized until above pH 7 (Abramson, Katzman, Wilson & Gregor, 1964).

In contrast, the introduction of a net positive charge on the lipid/water interface by the addition of stearylamine to the phosphatidylinositol (1:9 molar ratio) resulted in no enhancement of the penetration by the albumin even when this possessed a substantial negative charge. The explanation of this differential effect of the sign of the charge on the interface is obscure although it might be that the protonated amino groups can penetrate more readily than charged carboxyl groups. It is known that with increasing positive charge serum albumin acquires a more expanded structure (Tanford, 1965) but the effect would be minimal at pH 4.5. It has been observed that the binding of catalase (Fraser, Kaplan & Schulman, 1955) and trypsin (Fraser & Schulman, 1956) to oil-in-water emulsions was weak if the emulsion was stabilized with positively charged detergents and strong if negatively charged detergents were used. It may not be fortuitous that the phospholipids contained in biological membranes are either zwitterionic at a physiological pH or possess a net negative charge on their polar head groups (Dawson 1968).

The results might provide an explanation of the specific activation of certain phospholipases by anionic amphipathic substances. Of fundamental
consideration in enzymic reactions of this type is the possibility of collision and attachment of the enzyme about the bond undergoing hydrolysis. Many phospholipases e.g. phospholipases A, B and D, exhibit a marked lowering of activity towards yeast phosphatidylcholine monolayers when the pressure is higher than about 25–28 dyn/cm (Bangham & Dawson, 1960, 1962; Quarles & Dawson, 1969). The present results show that serum albumin does not penetrate into yeast phosphatidylcholine films at high surface pressures and similar results have previously been obtained with the interaction of cytochrome c with hen’s egg phosphatidylcholine monolayers (Quinn & Dawson, 1969b). A similar situation may occur with these phospholipases in which the enzyme cannot react with the monolayer at higher pressures. The introduction of appropriate charged molecules into the substrate may serve to attract and attach the enzyme to the surface as well as to facilitate penetration of the active centre to its site of action. These reactions are typified in the case of decapitation of phosphatidylcholine by a phospholipase of Penicillium notatum that specifically requires the introduction of negative charges on the phospholipid/water interface (Dawson, 1958, Bangham & Dawson, 1959); positive charges were not effective. The hydrolysis of monolayers of yeast phosphatidylcholine by the enzyme occurred at low pressures but at higher pressures only if an anionic amphipathic substance was added (Bangham & Dawson, 1960). Since it seems likely that hydrolysis of the phospholipid would require extensive penetration of the active centre on the enzyme to reach the fatty acyl ester bonds, it is probable that this would be assisted by the anionic amphipath. This would be similar to the electrostatically facilitated penetration of serum albumin into phosphatidylcholine monolayers above 20 dyn/cm.

It is not to be expected that all proteins would necessarily show the same degree of charge-assisted penetration characteristics as serum albumin. Preliminary experiments have shown that malate dehydrogenase also shows an electrostatically facilitated penetration into phosphatidylcholine monolayers when long-chain anions are added. However, melithin, a low-molecular-weight basic protein of bee venom penetrates phosphatidylcholine monolayers to the same extent irrespective of whether small amounts of dicetylpHosphoric acid or stearylamine are added (Sessa, Freer, Colacicco & Weissmann, 1969). Further, certain enzymes are only effective against positively charged phospholipid/water interfaces (Bangham & Dawson, 1962).

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REFERENCES