An Analysis of the Interaction of Protein with Lipid Monolayers at the Air/Water Interface

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1. Measurements have been made of the interaction of cytochrome *c*, bovine serum albumin and synthetic oxytocin with low-pressure (2 dyn/cm) monolayers of stearic acid, phosphatidylethanolamine and phosphatidylethanolamine. 2. [^{14}C]Carboxymethylation of the cytochrome *c* and albumin followed by surface-radioactivity determinations have shown that only a proportion of the protein added to the subphase is bound to the monolayers and that initially the degree of binding is dependent on the protein concentration. The binding is irreversible in the sense that the adsorbed protein cannot be removed by transferring the film containing the interacted protein to a fresh subphase containing no protein. 3. Three successive types of interaction can usually be recognized. (a) Initially, whole molecules of protein penetrate the lipid film and occupy the same area as those of the protein spread at the air/water interface. (b) Above certain film pressures a part of each protein molecule, probably hydrophobic side chains, penetrates the film. The change in surface pressure per unit of bound protein is much smaller than in (a). (c) At higher film pressures, adsorption without penetration occurs. With cytochrome *c* this is initially dependent on a favourable electrostatic interaction.

The interactions of protein with expanded lipid monolayers spread at the air/water interface have been observed by measuring the increment of surface pressure at constant area or the rate of expansion at constant pressure during the interaction. From such studies several methods by which the protein might associate with the lipid monolayer have been surmised. Thus Matalon & Schulman (1949) and Schulman (1957) consider the interaction to take place by a 'solution' of whole protein molecules in the film at low pressures followed by a penetration of the hydrophobic side chains of the amino acid residues when the film is initially above the collapse pressure of the protein at the air/water interface. In contrast, other workers (Eley & Hedge, 1956, 1957*a,b*; Eley & Miller, 1960) have suggested that whole protein molecules do not enter lipid monolayers even when the latter are at low surface pressures, but instead are adsorbed underneath the film. They suggested that each peptide bond of the protein is associated with the polar group of a lipid molecule through hydrogen and hydrophobic bonding although additional bonding sites have been postulated in the case of adsorption to phospholipid monolayers. This builds up an initial layer of expanded protein followed by the adsorption of a second layer more or less in a native conformation. Such an interpreta-

tion was favoured by Colacicco, Rapport & Shapiro (1967) in their investigations of the interaction of various proteins with monolayers of dihydroceramide lactosides, and Sanyal & Snart (1967) and Snart & Sanyal (1968) in their studies of the interaction of polypeptide hormones with monolayers of cholesterol and fatty acid.

The labelling of the interacting protein used in the present series of studies (Quinn & Dawson, 1969*a,b*) has introduced a new parameter into these observations. Thus by measuring the surface radioactivity one can determine precisely the surface concentration of reacting protein. This has already indicated that proteins can adsorb to phospholipid monolayers without producing an increment in the surface pressure (Quinn & Dawson, 1969*a*). In this investigation we have examined the interaction of labelled albumin and cytochrome *c* with low-pressure monolayers of stearic acid and phospholipids in an attempt to establish the nature of the interaction.

EXPERIMENTAL

Proteins. Bovine serum albumin was a crystalline preparation from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Fatty acids were removed by the procedure of Chen (1967). It was confirmed that this delipidated albumin gave an optical-rotatory-dispersion

spectrum almost identical with that of the commercial protein (an apparent 3% loss of helical content was within the limits of experimental error). The bovine serum albumin was relipidated in some experiments by allowing it to react in aqueous solution with an equimolar amount of [$1\text{-}^{14}\text{C}$]stearic acid.

Carboxymethylation of the albumin was carried out after reduction of some of the disulphide bonds (Hunter & McDuffie, 1959). The delipidated serum albumin (30 mg) was incubated with thioglycolic acid (60 mg) in 4 ml of 2M-urea. After the pH had been adjusted to 8.2 with 2M-tris, the mixture was left for 12 h at room temperature under N_2 . The presence of low concentrations of urea (2M) produces an increased susceptibility of a small proportion of the disulphide bonds to reduction (Kolthoff, Anastasi & Tan, 1959).

The protein was separated from the reactants by filtration in 0.1M-NaCl through a column (30 cm $\times$ 1.5 cm) of Sephadex G-25, care being taken to avoid reoxidation by contact with air. The protein-containing eluate (measured by E_{280}) was dialysed and then evaporated to dryness *in vacuo*. The partially reduced albumin (15 mg) was treated with 1.5 mg (250 μCi) of iodo[2- ^{14}C]acetic acid (The Radiochemical Centre, Amersham, Bucks., U.K.) in 2 ml of 50 mM-sodium acetate-acetic acid buffer, pH 4.5, and incubated for 20 h at 40°C in the dark under N_2 . The carboxymethylated albumin was then recovered by gel filtration in 0.1M-NaCl through a column (30 cm $\times$ 1.5 cm) of Sephadex G-25 and the protein peak (E_{280} corresponding to the radioactivity) was collected, dialysed, evaporated to dryness *in vacuo* and made up in water to a concentration of 100 $\mu\text{g/ml}$. Any unreacted sulphhydryl groups were allowed to oxidize by exposure to air. The specific radioactivity was 15.5 mCi/mmol; calculation showed that this represented an average of approx. one carboxymethylated sulphhydryl group per molecule. Reduction under the conditions used is unlikely to cause any change in the molecular weight or conformation of the albumin (Hunter & McDuffie, 1959; Kolthoff *et al.* 1959). In certain experiments in which measurements were made of the area occupied by serum albumin at the air/water interface the protein was not reduced before reaction with iodo[2- ^{14}C]acetate. This gave a protein of low specific radioactivity which, although suitable for these experiments, was unsatisfactory for measuring adsorption on to lipid/water interfaces.

Cytochrome *c* was carboxymethylated with iodo[2- ^{14}C]acetic acid as described by Quinn & Dawson (1969a). The specific radioactivity was 11.4 mCi/mmol, indicating that about one carboxymethyl group had been introduced into each protein molecule.

Synthetic oxytocin free of preservative was generously provided by Dr B. Berde, Sandoz Products Ltd. (Pharmaceutical Dept.), London W.1, U.K. Attempts to use the commercially available synthetic peptide led to completely spurious results due to the surface activity of a preservative, chlorbutol, present in these preparations.

Lipids. The preparation and purification of the phospholipids used was as described by Quinn & Dawson (1969a,b). The stearic acid was a commercial sample (British Drug Houses Ltd., Poole, Dorset, U.K.) recrystallized twice from ethanol and twice from benzene. It gave a single peak on g.l.c.

Apparatus and techniques. The techniques used for

Fig. 1. Relationships between surface radioactivity and surface concentration of radioactive proteins. Subphase, 50% satd. $(\text{NH}_4)_2\text{SO}_4$. ●, [^{14}C]Carboxymethylated albumin; ○, [^{14}C]carboxymethylated cytochrome *c*.

spreading the monolayers of lipid and for continuously measuring and recording the surface radioactivity, pressure and potential on allowing them to interact with protein were as described by Quinn & Dawson (1969a,b). In some experiments involving the transfer of a monolayer containing interacted protein to a fresh subphase the trough was divided into two compartments by the introduction of a Fluon division which came to within 1 mm of the surface monolayer. The monolayer was transferred to the fresh subphase by sliding it between two barriers kept the same distance apart to avoid any pressure changes in the film during transposition.

Correlation of surface radioactivity with number of molecules of protein per cm^2 of interface. The surface-radioactivity technique measures all labelled molecules concentrating in the surface phase, and is determined as the increment of radioactivity above that of the background of the bulk radioactivity in free solution in the trough. By using a weak β -emitter effective shielding from labelled molecules in the subphase is obtained. Calibration of the counter to measure directly the number of molecules of protein per cm^2 of interface was carried out by fixing the window of the counter at a set distance from the surface of the trough filled with 50% saturated $(\text{NH}_4)_2\text{SO}_4$. The protein was introduced on to the surface in successive portions from an Agla syringe and the subphase was stirred for 10 min; the surface radioactivity was continuously recorded. The average surface radioactivity and protein concentration were directly proportional over the range examined (Fig. 1). The molecular weights used for calculating the number of molecules of protein per cm^2 at the interface were 12363 for cytochrome *c* (Margoliash & Schejter, 1966) and 67000 for bovine serum albumin (Phelps & Putnam, 1960).

RESULTS

Interaction between bovine serum albumin and its fatty acid complex with monolayers of stearic acid. When commercial bovine serum albumin containing

a small proportion of complexed fatty acid (Chen, 1967) was allowed to interact with a stearic acid film at an initial pressure of 2 dyn/cm, the increment in surface pressure ($\Delta\pi$) exhibited a biphasic relationship with the concentration of protein added (Fig. 2). The inflexion points, which were independent of subphase stirring, correspond closely to those observed by Eley & Hodge (1957a), who used a similar unstirred system except that they measured the surface pressure by means of a Langmuir horizontal surface balance rather than a Wilhelmy dipping device.

When bovine serum albumin had been treated with activated charcoal at low pH to remove complexed fatty acids (Chen, 1967) much larger values were obtained (Fig. 2). The $\Delta\pi$ -protein-concentration plots showed some difference between the unstirred and stirred systems although the maximum $\Delta\pi$ values achieved were identical for both (Fig. 2). The curves suggest that in the absence of stirring the albumin added to the subphase does not come into true equilibrium with the film. Presumably the reaction may still be occurring at a very slow rate that is governed by the rate of diffusion of protein molecules to the interface and that is not detectable by a change in surface pressure over the time period studied. Presumably the greater $\Delta\pi$ values observed with delipidated albumin could be due to the exposure of hydrophobic residues on the protein surface. Measurements with the apoprotein of high-density plasma lipoprotein have emphasized that hydrophobic interactions can greatly assist the penetration of proteins into lipid monolayers even

at well above the collapse pressure of the protein itself (Camejo, Colacicco & Rapport, 1968). Unlike the latter authors, however, we have found that both the fatty-acid-complexed and lipid-free serum albumin are surface active and collect at the interface when added to the bulk phase; this conforms with the known surface activity of many other proteins.

If the delipidated albumin was complexed with fatty acid ($[1-^{14}\text{C}]$ stearic acid) before the interaction with the stearic acid monolayer, the $\Delta\pi$ -protein-concentration curve became similar to that shown by the original commercial preparation except that it was difficult to distinguish any biphasic relationship. Determination of the surface radioactivity showed that the amount of complex interacting with the film was equivalent to the $\Delta\pi$ values observed. However, calculation of the amount of label added indicated that only a small percentage (approx. 10%, cf. Table 2) of the complexed protein had interacted with the monolayer at any concentration in the subphase, assuming that the complex had not dissociated into its components. Additional calculations showed that an exchange of stearic acid molecules between the monolayer and the complex was unlikely.

Interaction between $[^{14}\text{C}]$ carboxymethylated albumin and lipid monolayers. Since these results, combined with those of previous investigations using cytochrome *c* (Quinn & Dawson, 1969a,b), had indicated that proteins do not react quantitatively when injected under low-pressure monolayers of lipids, more precise quantitative studies were next undertaken with specifically labelled proteins.

Figs. 3, 4 and 5 show the interaction of $[^{14}\text{C}]$ carboxymethylated albumin with monolayers of

Fig. 2. Interaction of albumin (native and delipidated) and stearic acid monolayers at 2 dyn/cm. Subphase, 40 mM-phosphate buffer, pH 7.4, + NaCl to 150 milliosmolar. Surface-pressure changes, $\Delta\pi$. Delipidated albumin: ●, stirred subphase; ○, unstirred subphase. Commercial albumin (with fatty acid): ■, stirred subphase; □, unstirred subphase. Relipidated albumin (1 mol of $[1-^{14}\text{C}]$ stearic acid/mol of protein), stirred subphase: △, change in surface pressure ($\Delta\pi$); ▲, surface radioactivity (ΔR).

Fig. 3. Changes in surface pressure, radioactivity and potential on adding $[^{14}\text{C}]$ carboxymethylated albumin below a monolayer of stearic acid at 2 dyn/cm. Subphase, 40 mM-phosphate buffer, pH 7.4, + NaCl to 150 milliosmolar; stirred. Each point represents an individual monolayer of approx. 80 cm². ●, Change in surface pressure ($\Delta\pi$); ○, change in surface $[^{14}\text{C}]$ protein; □, change in surface potential (ΔV).

Fig. 4. Changes in surface pressure, radioactivity and potential on adding [^{14}C]carboxymethylated albumin below a monolayer of phosphatidylethanolamine at 2 dyn/cm. Subphase, 40 mM-phosphate buffer, pH 7.4, + NaCl to 150 milliosmolar; stirred. ●, Change in surface pressure ($\Delta\pi$); ○, change in surface [^{14}C]protein; □, change in surface potential (ΔV).

Fig. 5. Changes in surface pressure, radioactivity and potential on adding [^{14}C]carboxymethylated albumin and delipidated albumin below monolayers of phosphatidylcholine at 2 dyn/cm. Subphases: [^{14}C]albumin, 40 mM-phosphate buffer, pH 7.4, + NaCl to 150 milliosmolar; delipidated albumin, 10 mM-NaCl. Change in surface pressure ($\Delta\pi$): ●, [^{14}C]carboxymethylated albumin; △, delipidated albumin. Change in surface [^{14}C]protein; ○, Change in surface potential (ΔV); □, [^{14}C]carboxymethylated albumin; ■, delipidated albumin.

stearic acid, phosphatidylethanolamine and phosphatidylcholine at an initial pressure of 2 dyn/cm. With the phospholipids the $\Delta\pi$ -protein-concentration curves clearly show an inflexion point before the maximum pressure increment is reached. The relationship between the increase in surface pressure of phosphatidylcholine monolayers and the protein concentration with the ^{14}C -labelled albumin differed from that with the delipidated albumin (Fig. 5). However, this difference might be accounted for by the fact that the reactions were carried out on subphases containing different concentrations of salt rather than there being intrinsic differences between the labelled and non-labelled protein molecules. High salt concentration could cause a contraction of the albumin molecules at the interface, although clearly this does not

happen with cytochrome *c* (Fig. 11c, Quinn & Dawson, 1969b). The inflexion points were reflected in similar changes in the surface radioactivity-protein-concentration curve expressed in the figures as molecules of radioactive albumin associated with 1 cm^2 of the surface film.

With stearic acid monolayers the first inflexion point in the $\Delta\pi$ -albumin-concentration plot is not so immediately obvious as with the phospholipid monolayers (Fig. 3). However, breaks in the ΔV - and surface-radioactivity-protein-concentration plots are apparent and have been used to construct a two-slope line for the $\Delta\pi$ plot.

In every instance the binding of ^{14}C -carboxymethylated albumin to the monolayer increased with increased protein concentration after penetration had ceased and $\Delta\pi$ was stable (Figs. 3, 4 and 5). In a previous investigation we have shown that a similar adsorption of [^{14}C]cytochrome *c* on to acidic phospholipids occurs after equilibrium penetration and this was ascribed to electrostatic interaction with the oppositely charged head-groups of the phospholipid (Quinn & Dawson, 1969a). Since the prevailing electrostatic conditions in the present experiments would be expected to preclude such an interaction, it is possible that protein-protein interactions between the bulk and penetrated protein are operating. Even with the zwitterionic phosphatidylcholine monolayers this non-penetrating binding was observed whereas a similar adsorption was not shown by cytochrome *c* (Quinn & Dawson, 1969b). In the latter instance protein-protein interactions were minimal.

*Interaction between [^{14}C]carboxymethylated cytochrome *c* and lipid monolayers.* The interaction of [^{14}C]cytochrome *c* with stearic acid monolayers again gave clear evidence of two inflexion points in the $\Delta\pi$ -protein-concentration plot (Fig. 6). The first was associated with corresponding inflexion points in the surface radioactivity and potential. The protein continued to bind to the monolayer after further penetration had ceased and $\Delta\pi$ had become stable. This is presumably due to the opposite charges on the protein and stearic acid at the pH used (7.4) resulting in non-penetrating electrostatic binding (Quinn & Dawson, 1969a,b). This type of binding was not seen with phosphatidylcholine monolayers (Fig. 7) where the polar head would possess no net charge at this pH (Bangham & Dawson, 1959). Whereas the surface radioactivity-cytochrome *c*-concentration plot showed a clearly biphasic pattern, the $\Delta\pi$ -protein-concentration curve appeared to fall off at first becoming linear at the point of inflexion of the previous plot (Fig. 7). A similar $\Delta\pi$ -protein-concentration curve was obtained with native cytochrome *c* and phosphatidylcholine monolayers except that the penetration at lower pressures

Fig. 6. Changes in surface pressure, radioactivity and potential on adding [^{14}C]carboxymethylated cytochrome *c* below a monolayer of stearic acid at 2 dyn/cm. Subphase, 40mM-phosphate buffer, pH 7.4, +NaCl to 150 milliosmolar; stirred. ●, Change in surface pressure ($\Delta\pi$); ○, change in surface [^{14}C]protein; □, change in surface potential (ΔV).

Fig. 7. Changes in surface pressure, surface potential and protein concentration in the surface phase on adding [^{14}C]carboxymethylated cytochrome *c* below a monolayer of phosphatidylcholine at an initial pressure of 2 dyn/cm. Subphase, 10mM-NaCl. ●, Change in surface pressure ($\Delta\pi$); ○, change in surface [^{14}C]protein; □, change in surface potential (ΔV).

appears to be enhanced by the carboxymethylation involved in the labelling (Quinn & Dawson, 1969b). However, the maximum pressures reached by the penetration of the native and labelled proteins were identical (16 dyn/cm).

Although the $\Delta\pi$ -cytochrome *c*-concentration curve for phosphatidylethanolamine monolayers has been constructed with an inflexion point (Fig. 8) this is certainly less convincing than with many of the interactions. Nor does the change in surface potential cease at the first inflexion point as observed with the other interactions. In our previous investigation (Quinn & Dawson, 1969a) we found no indication of a biphasic relationship when native cytochrome *c* penetrated phosphatidylethanolamine films from a subphase of 10mM-sodium chloride.

Interaction between oxytocin and stearic acid monolayers. The increments in surface pressure on adding oxytocin below stearic acid monolayers initially at

Fig. 8. Changes in surface pressure, surface potential and protein concentration in the surface phase on adding [^{14}C]carboxymethylated cytochrome *c* below a monolayer of phosphatidylethanolamine at an initial pressure of 2 dyn/cm. Subphase, 40mM-phosphate buffer, pH 7.4, +NaCl to 150 milliosmolar. ●, Change in surface pressure ($\Delta\pi$); ○, change in surface [^{14}C]protein; □, change in surface potential (ΔV).

Fig. 9. Changes in surface pressure on the addition of oxytocin to the subphase below stearic acid monolayers at 2 dyn/cm. □, Water subphase. 10mM-NaCl subphase: ●, stirred; ○, unstirred.

2.0 dyn/cm are shown in Fig. 9. Some differences were observed in the relationship between penetration and concentration with subphases of water or 10mM-NaCl and whether they were stirred or unstirred, but the maximum $\Delta\pi$ values observed were always the same (7 dyn/cm). These curves are in marked contrast with the values presented by Snart & Sanyal (1968) for the molar relationship between stearic acid and oxytocin at the point of maximum $\Delta\pi$ obtained while investigating a similar system. Thus, whereas the present results give a stearate/added oxytocin ratio of 0.3 at $\Delta\pi_{\text{max}}$, those of Snart & Sanyal (1968) suggest a ratio of approx. 6 at this point.

DISCUSSION

It is reasonably certain that the procedures used for labelling cytochrome *c* and bovine serum albumin cause limited changes in the properties

Fig. 10. (a) Plot of surface pressures (π) and radioactivities (ΔR) of [^{14}C]carboxymethylated non-reduced albumin against area occupied per mg of protein. Subphase, 40mM-phosphate buffer, pH 7.4, + NaCl to 150 millimolar. The lower curves 1 and 2 (O) are the surface radioactivities corresponding to the surface-pressure curves 1 and 2 above. The lower curve (●) refers to the surface radioactivity of the protein spread on a subphase 50% satd. with respect to $(\text{NH}_4)_2\text{SO}_4$. (b) Plot of surface pressure and surface radioactivity of a [^{14}C]carboxymethylated reduced albumin monolayer against the area per molecule. Subphase as for (a). —, Surface pressure (π); ----, surface radioactivity (ΔR).

of the proteins and some alteration in the way in which these react with lipid monolayers. The force-area curves of native and carboxymethylated cytochrome *c* at the air/water interface, however, are very similar (Fig. 12) and this also applies to their optical-rotatory-dispersion spectra (Mirsky & George, 1967), but some difference has been reported in their penetration into expanded phosphatidylcholine monolayers (Quinn & Dawson, 1969b)

although not into films of phosphatidylethanolamine (Quinn & Dawson, 1969a). Furthermore, it has been reported that serum albumin can exist at the air/water interface in two forms, one in which the molecules are expanded and the other in which they are more condensed and presumably closer to the configuration of the protein in free solution (Mussellwhite & Palmer, 1968). This has been confirmed unambiguously by spreading a comparatively large amount of [^{14}C]carboxymethylated serum albumin (non-reduced before carboxymethylation to avoid changes in conformation) on a limited interfacial area ($0.54\text{m}^2/\text{mg}$). A force-area curve was prepared by compression (Fig. 10a, curve 2). The experiment was repeated with a smaller amount of protein ($2.0\text{m}^2/\text{mg}$) giving a force-area curve that was quite different from the previous one (Fig. 10a, curve 1). The two types of curve were also obtained if the protein was initially spread at the higher density and then expanded greatly and allowed to stand for some time with subphase stirring before recompression. Radioactivity measurements showed that there was no loss of protein from the surface (Fig. 10a). Although the reason for this difference in the $\Delta\pi$ -area curves at different initial spreading areas is not completely understood we have assumed in our subsequent calculations that due to the pressure constraints imposed by penetration into lipid monolayers the protein would enter in the condensed form. The curve obtained by spreading the [^{14}C]carboxymethylated reduced albumin at the air/water interface in this condensed form is given in Fig. 10(b). This force-area curve differs only slightly from that of the unlabelled delipidated serum albumin spread under the same conditions, with the latter appearing to be somewhat more expanded.

In spite of differences between native and labelled proteins the latter can still be used as satisfactory models for understanding the mechanisms by which proteins can react with lipid monolayers. The surface-radioactivity technique permits a definite conclusion to be reached regarding the nature of the interaction of protein with low-pressure lipid monolayers and provides an explanation for the biphasic $\Delta\pi$ -protein-concentration curves obtained. From the force-area curves of the lipids used (Fig. 11) it is possible to calculate from a geometric model (Pethica, 1955) the area made available by compression of the films from the initial starting pressure of 2 dyn/cm to the pressure of the first and second inflexion points of the interacted protein-lipid film shown in Figs. 3, 4, 5, 6, 7 and 8. Likewise from the force-area curves of the carboxymethylated proteins at the air/water interface (Figs. 10 and 12) and the number of molecules of protein reacting with the film (deduced from surface-radioactivity determinations) the area

Fig. 11. Relationship between surface pressure and area per molecule of lipid monolayers. Subphase, 40mM-phosphate buffer, pH 7.4, +NaCl to 150 milliosmolar. (a) Stearic acid; (b) phosphatidylcholine (egg); (c) phosphatidylethanolamine (egg).

that would be occupied by the interacted protein, assuming its conformation was the same as at the air/water interface, can be calculated. In spite of the limitations in employing the geometric model (Eley & Hedge, 1957b) the two values show reasonably good agreement considering the variety of experimental observations used in their derivation (Table 1). This indicates that, as originally suspected by Schulman (1957), the protein molecules in their entirety are entering gaps in the expanded lipid films and are occupying spaces similar to those of the condensed protein at the air/water interface. Also, it should be noted that the first inflexion point is always at a pressure below the collapse pressure of the protein at the air/water interface. After the first inflexion point this relationship no longer holds and it is apparent that more protein is reacting with the film than could be accounted for by whole protein molecules entering the available space in the monolayer (Table 1). It seems likely that in such circumstances only a part of the protein molecule is penetrating the film; the hydrophobic side chains of the non-polar amino acid residues are obvious candidates for this role.

A third type of interaction between the components is shown by the increase in surface radioactivity without any change in surface pressure. This implies that additional protein molecules are reacting with the lipid film already containing penetrated protein molecules. Although there is a possibility that this could be a protein-protein interaction we have shown (Quinn & Dawson,

Fig. 12. Relationship between the surface pressure and area per molecule of cytochrome *c* and [^{14}C]carboxymethylated cytochrome *c* monolayers. (a), Cytochrome *c*, subphase 40mM-phosphate buffer, pH 7.4, +NaCl to 150 milliosmolar; (b), cytochrome *c*, subphase 10mM-NaCl; (c), [^{14}C]carboxymethylated cytochrome *c*, subphase 10mM-NaCl.

1969a,b) that cytochrome *c* can react with high-pressure lipid monolayers at pressures higher than those at which protein penetration is possible. With cytochrome *c* this type of reaction seems to be entirely dependent initially on electrostatic forces, e.g. no adsorption has been observed on zwitterionic phosphatidylcholine monolayers or to negatively charged films in the presence of high salt concentrations.

Initially on addition of protein to the subphase the energy required for whole protein molecules to enter the film and orientate themselves at the interface would be comparatively low because of the greatly expanded nature of the monolayer, but as the pressure increases, a point will be reached when the energy requirement precludes whole-molecule penetration, and only side-chain penetration with its lower energy needs (Haydon & Taylor, 1963) is possible. It is conceivable that in some interactions there would be an overlap between these two types of penetration so that the inflexion in the $\Delta\pi$ -protein-concentration curve would not be distinct.

Whether or not whole protein molecules leave the lipid film after the first inflexion point probably depends both on the nature of the protein and on the forces between it and the adjacent lipid molecules. With cytochrome *c*, any conformational change occurring at the interface would be expected to be reversible (Reich & Waino, 1961; Myer, 1968) and consequently it is found that the protein is desorbed from phospholipid monolayers on compression when there is no electrostatic interaction. For example, cytochrome *c* is ejected from monolayers of phosphatidylcholine but not from the negatively charged phosphatidylethanolamine unless high salt concentrations are present in the subphase (Quinn & Dawson, 1969a,b).

Table 1. *Stoichiometric relationship between proteins and lipids interacting at the air/water interface*

A geometric method was used to compare the area occupied by the protein and the theoretical decrease in area of the lipid molecules at the interface.

Protein	Lipid	First inflexion point			Changes between first and second inflexion points		
		$\Delta\pi$ (dyn/cm)	$10^{14} \times$ Area of protein ($\text{\AA}^2/\text{cm}^2$)	$10^{14} \times$ De- crease in area of lipid ($\text{\AA}^2/\text{cm}^2$)	$\Delta\pi$ (dyn/cm)	$10^{14} \times$ Area of protein ($\text{\AA}^2/\text{cm}^2$)	$10^{14} \times$ De- crease in area of lipid ($\text{\AA}^2/\text{cm}^2$)
[^{14}C]Carboxymethylated cytochrome <i>c</i>	Stearic acid	6.4	7.8	6.0	3.6	4.4	2.0
	Phosphatidylcholine	10.8	25.4	19.4	5.2	21.3	4.0
	Phosphatidyl- ethanolamine	10.8	23.1	22.0	2.0	29.4	3.4
[^{14}C]Carboxymethylated albumin	Stearic acid	4.1	5.1	4.3	3.0	9.3	0.8
	Phosphatidylcholine	8.1	18.6	16.5	2.9	28.9	2.9
	Phosphatidyl- ethanolamine	8.5	16.4	17.8	2.6	17.8	5.1

Table 2. *Percentage of added protein bound to lipid monolayers*

Protein	Lipid	First inflexion point	Second inflexion point
[^{14}C]Carboxymethylated cytochrome <i>c</i>	Stearic acid	74.0	26.0
	Phosphatidylcholine	10.0	2.1
	Phosphatidylethanolamine	26.0	18.8
[^{14}C]Carboxymethylated albumin	Stearic acid	20.5	15.4
	Phosphatidylcholine	19.4	11.4
	Phosphatidylethanolamine	14.0	6.6

It seems possible, therefore, that after the first inflexion point in the $\Delta\pi$ -protein-concentration curve whole cytochrome *c* molecules leave the phosphatidylcholine monolayers and immediately become bonded through side-chain penetration. With other proteins that are more prone to irreversible denaturation it is likely that whole protein molecules may remain in the monolayer as the pressure rises above the first inflexion point in the $\Delta\pi$ -protein-concentration curve. Albumin, for example, when it interacts with a film of phosphatidylcholine can only be partially ejected by compression of the monolayer to high pressures. Thus, between the first and second inflexion points the lipid film contains whole protein molecules and there are protein molecules underneath with their side chains penetrating into the mixed film. This would explain why the surface pressures reached in such systems are sometimes well above the collapse pressure of the protein at the air/water interface, an observation that has often been used as an argument against the entry of whole protein molecules into lipid monolayers (Hedge, 1957; Colacicco *et al.* 1967).

The technique has also revealed the surprising result that, when protein is added under a lipid monolayer, only a proportion of the added protein reacts with the film (Table 2) and that the amount reacting is dependent on the concentration of protein added. Similar results were obtained in our investigation of the interaction of cytochrome *c* with phosphatidylethanolamine monolayers (Quinn & Dawson, 1969a).

The evidence for the incomplete reaction of the protein with the monolayer was mainly derived by calculation of the amount of protein in the surface (deduced from surface-radioactivity measurements) and comparison with the amount added (Table 2). In initial experiments, the trough itself bound a small proportion of the added protein which was not removed by the washing procedure employed. In subsequent experiments, however, the radioactivity not associated with the monolayer could all be recovered in samples of the bulk phase taken from under the film. Moreover, the incompleteness of the interaction could be demonstrated by other techniques with the native proteins. Thus, on sweeping off the monolayer of lipid containing

Table 3. *Changes in surface pressure on spreading successive monolayers of stearic acid on to a subphase containing albumin*

Subphase: 40 mM-sodium phosphate buffer, pH 7.4, + NaCl to 150 milliosmolar.		Surface pressure (dyn/cm)			
Expt. no.	Protein added	Initial lipid/protein molar ratio in system	Monolayer	Initial π	Increment at equilibrium $\Delta\pi$
1	Native (3.3 μ g)	585	1st	2.0	4.4
			2nd	2.0	4.0
2	Delipidated (7.3 μ g)	305	1st	2.0	11.8
			2nd	1.9	12.8

Table 4. *Effect of removing protein from the subphase at equilibrium on protein penetrated into or adsorbed on to phospholipid monolayers*

Expt. no.	Phospholipid monolayer	Protein	Surface measurement	Pressure (dyn/cm)	Radioactivity (c.p.s. — background)
1	Phosphatidylcholine	[14 C]Carboxymethylated cytochrome <i>c</i>	Initial	2.1	0
			Final at equilibrium	12.2	35
			Transposed film after 45 min	12.5	31
2	Phosphatidylcholine	Delipidated albumin	Initial	2.0	—
			Final at equilibrium	17.1	—
			Transposed film after 45 min	17.0	—
3	Phosphatidylethanolamine	[14 C]Carboxymethylated cytochrome <i>c</i>	Initial	30.2	0
			Final at equilibrium	30.2	29
			Transposed film after 45 min	27.0	26

interacted protein (at equilibrium) between two barriers (see the Experimental section), surface-active material accumulated at the interface. If a new lipid film was spread on such a freshly exposed subphase the eventual increment in surface pressure and potential was approximately equal to that found with the initial film (Table 3). Similar results were also obtained with oxytocin although experiments were not performed with a radioactive derivative of the polypeptide. These results are to be expected only if a small amount of the protein added had interacted with the initial film.

It is often assumed that protein added under low-pressure lipid films reacts quantitatively with the lipid, and this has been used to calculate stoichiometric relationships between the lipid and protein and to build molecular models of the lipoprotein complexes formed. The present results emphasize that considerable caution must be exercised in accepting the conclusions reached by such calculations.

Although the amount of protein that interacted with the monolayer was clearly related to the con-

centration of protein added, there was no evidence that the reaction was reversible when the protein was removed from the bulk phase of a film at equilibrium. Thus when [14 C]cytochrome *c* interacted with a low-pressure monolayer of phosphatidylcholine and was then transferred to a fresh subphase not containing protein and maintaining the same area there was no loss of film pressure, nor significant decrease in surface radioactivity (Table 4). Similar results have also been obtained with [14 C]cytochrome *c* and monolayers of phosphatidylethanolamine (Quinn & Dawson, 1969a). Moreover, with high-pressure phosphatidylethanolamine films, where no penetration of [14 C]cytochrome *c* is possible, the adsorption of protein was again irreversible since no significant loss of surface radioactivity was observed on transferring the film at constant area to a fresh subphase (Table 4). Although the reversibility of the reaction of albumin with lipid monolayers was not investigated with the [14 C]carboxymethylated preparation, the maintenance of the same pressure on transferring the penetrated protein to a fresh subphase again

indicates that it is substantially irreversible. The use of radioactive proteins has enabled us to test a modification of the Gibbs equation that expresses changes in the pressure of monolayers in terms of the surface concentration of the penetrating species in the presence of excess of electrolytes (Pethica, 1955). This has been used by Eley & Hedge (1957b) to quantitatively determine the penetration of various proteins into lipid monolayers. The calculated values were always much higher than the observed protein concentration at the interface. Failure to find agreement in protein-lipid interactions at the air/water interface may be related to the apparent irreversibility of the interaction, since the derivation of the Gibbs equation assumes reversibility of adsorption.

It appears therefore that the protein concentration in the subphase in some way determines the extent to which the energy barriers preventing penetration and bonding of the protein to the interface are overcome. Once the interaction has taken place the protein-lipid complex does not dissociate. Thus the reaction apparently does not depend on the protein reacting with a fixed number of binding sites, which are filled provided protein is left in the subphase and collisions are still occurring rendering the protein insoluble.

That denaturation does not account for the irreversibility is indicated by the observation that cytochrome *c* can be expelled from phosphatidylcholine films by compressing the film to only moderately high pressures (35 dyn/cm). Further, it is clear that the protein that is interacting does not differ from that remaining in the bulk phase, since penetration can still occur almost to the same extent if another monolayer is spread at the same initial pressure (Table 3).

Although the factors that control the dependency of the bound protein on the protein concentration in the subphase are not understood, it is apparent that with cytochrome *c* at least the extent of the binding is increased by favourable electrostatic conditions. Thus the positively charged protein is bound to a lesser extent to the zwitterionic phosphatidylcholine than to stearic acid (Table 2), whereas the bonding to films of high negative charge density e.g. phosphatidic acid, is virtually quantitative (Quinn & Dawson, 1969b). The carboxymethylated albumin would be negatively charged at the pH of the system (7.4) and this would explain why it reacts more readily with phosphatidylcholine than

with phosphatidylethanolamine. On the other hand the interaction with a stearic acid monolayer is greater than expected. Clearly the factors that control the distribution are complex and are probably also influenced by non-ionic interactions.

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