The Interaction of Cytochrome *c* with Monolayers of Phosphatidylethanolamine

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1. The interaction between [^{14}C]carboxymethylated cytochrome *c* and monolayers of egg phosphatidylethanolamine at the air/water interface has been investigated by measurements of surface radioactivity, pressure and potential. 2. On adding ^{14}C -labelled cytochrome *c* to the subphase under monolayers with a surface pressure below 24 dynes/cm. there was an initial surface pressure increment as the protein penetrated, followed by an adsorption that could be detected only by a continued increase in the surface radioactivity. 3. Above film pressures of 24 dynes/cm. only adsorption was observed, i.e. an increment in surface radioactivity with none in surface pressure. 4. The changes in surface parameters with penetration of cytochrome *c* added to the subphase were indirectly proportional to the initial pressure of the monolayer. With hydrogenated phosphatidylethanolamine the constant of proportionality was increased but penetration again ceased at 24 dynes/cm. 5. On compressing a phosphatidylethanolamine film containing penetrated cytochrome *c* to 40 dynes/cm. only a proportion of the protein was ejected on a subphase of 10 mM-sodium chloride, whereas on a subphase of M-sodium chloride nearly all the protein was lost. 6. With both penetration and adsorption only a small proportion of the added cytochrome *c* interacted with the phospholipid films, and initially the amount bound was proportional to the added protein concentration. There was no evidence of a stoichiometric relationship between the protein and phospholipid or the build-up of multilayers. The bonded protein was not released by removing cytochrome *c* from the subphase. 7. The addition of M-sodium chloride to the subphase delays the rate of protein penetration into low-pressure films, but the final surface-pressure increment is not appreciably decreased. In contrast, M-sodium chloride almost completely stops adsorption on to films at all pressures. 8. When sodium chloride is added to the subphase below cytochrome *c* adsorbed to monolayers at high pressures, so that the final concentration is 1 M, only a proportion of the protein is desorbed and this decreases as the time of the interaction increases. This indicates that adsorption is initially electrostatic, followed by the formation of non-ionic bonds. 9. Alteration of the subphase pH under a high-pressure film leads to a steady increase in adsorption from pH 3 to 8.5 followed by a rapid fall to zero adsorption at pH 11. 10. The penetration into phospholipid monolayers at 10 dynes/cm. shows a rate that is consistent with the relative electrostatic status of the two components of the interaction as the subphase pH is varied between 3 and 10.5. The final equilibrium penetration shows a pronounced peak in the increments of surface pressure at pH 9.0 although a similar peak is not observed in the surface radioactivity. This indicates that more residues of the protein are penetrating into the film at about this pH. 11. Determinations were made of the electrophoretic mobilities of phosphatidylethanolamine particles both alone and after interaction with cytochrome *c*. 12. The electrophoretic mobilities of cytochrome *c* adsorbed on lipid particles showed an isoelectric point below that of cytochrome *c*. This and the observations on the monolayers suggest that, with cytochrome *c*, protein-protein interactions are weak compared with other proteins.

The bonding between the lipid and protein moiety of natural lipoproteins is essentially non-covalent but the precise type of bonding involved is imper-

fectly understood. One of the few methods available for investigating this type of interaction is that of adding a soluble protein to a subphase below a

unimolecular film of lipid orientated at the air/water interface. Such a lipid film has often been cited as a basic structural unit of both the micellar and bimolecular leaflet form of the lipid and it has the advantage that the spacing between the lipid molecules can be readily varied by altering the area of the film by compression or expansion. However, previous studies using this technique have been limited by the difficulties inherent in determining whether binding of protein to the lipid film has occurred. Detection of such a binding has been carried out by measuring an increment in the surface pressure of the film (e.g. Doty & Schulman, 1949; Colacicco, Rapport & Shapiro, 1967) or in the special case of phospholipases and their substrates by the enzymic hydrolysis and disintegration of the monolayer (Hughes, 1935; Bangham & Dawson, 1960). Unfortunately the increment in surface pressure, which involves a physical penetration of at least a part of the protein molecule into the film, occurs only at low film pressures, i.e. when there is a low packing density of the lipid molecules at the interface. It is by no means certain that this type of bonding is involved in the various types of protein-lipid interactions that occur in Nature, e.g. membrane formation, interaction of antibodies with lipid antigens, blood clotting and enzyme activation. In fact, present evidence tends to suggest that the density of packing of phospholipids in micelles and bimolecular leaflets is well above the level where protein penetration into a unimolecular film of lipid typically ceases.

In the present investigation the bonding between a soluble protein and a unimolecular film of phospholipid has been studied not only by the conventional techniques of measuring changes in the surface pressure and interfacial potential but also by labelling the protein with a weak β -emitter and determining the surface radioactivity. An increase of the latter above the background radioactivity obtained when the same amount of radioactivity is evenly distributed throughout the aqueous medium in the trough indicates that the protein molecules have moved towards the surface phase (Salley, Weith, Argyle & Dixon, 1950). In other words, this asymmetry of protein distribution implies that bonding of the protein to the surface has occurred.

For our initial studies we have chosen the interaction of cytochrome *c* with phosphatidylethanolamine. There are a number of reasons for this choice. First, cytochrome *c* is a readily soluble protein, resistant to denaturation and of known primary structure so that the hydrophobic regions have been mapped (Margoliash & Schejter, 1966). Secondly, the carboxymethylation that was used as a means of introducing the ^{14}C label into this protein is well documented (Ando, Matsubara &

Okunuki, 1966*a,b*) and under certain circumstances hardly affects the biological activity. Thirdly, the lipid-protein reaction itself has been well studied in the bulk phase by using phosphatidylethanolamine particles and measuring as an index of reaction the precipitation of the protein or its extractibility into organic solvents (Reich & Waino, 1961; Das, Myers & Crane, 1964; Das, Haak & Crane, 1965). In fact, such 'complexes' have been used by Green & Fleischer (1963) as models to discuss the form of binding of cytochrome *c* in intact mitochondria. In this they assumed that the bonding is exclusively electrostatic in nature. The present investigation finds that such ionic bonding is of importance in the interaction between cytochrome *c* and phosphatidylethanolamine but also indicates that non-ionic forms of bonding can occur.

METHODS

[^{14}C]Carboxymethylation of cytochrome *c*. Cytochrome *c* (0.162 μmole) from horse heart (Koch-Light Laboratories Ltd., Colnbrook, Bucks.; 0.42% Fe determined by the method of Cameron, 1965) was treated at pH 3.5 with iodo[2- ^{14}C]acetic acid (24.7 μmoles ; 15.5 mc/m-mole ; The Radiochemical Centre, Amersham, Bucks.) by the procedure outlined by Ando *et al.* (1966*a,b*). Iodoacetate was chosen as the reagent since it reacts more readily with cytochrome *c* than does iodoacetamide (Ando *et al.* 1966*a,b*). The carboxymethylation according to these authors proceeds mainly on methionine-65 and methionine-80 at low pH values. The carboxymethylated cytochrome *c* was purified by Sephadex G-25 gel filtration as described except that this had been equilibrated with 0.1 M-NaCl rather than with phosphate buffer: the radioactive peak (corresponding with the absorption peak at 550 nm.) emerged immediately and was collected and diluted with distilled water to a final concentration of 0.0365 $\mu\text{mole/ml.}$ (450 $\mu\text{g./ml.}$). The carboxymethylated cytochrome *c* was stored frozen; on storage at 4° the product as well as the native protein showed a change in its behaviour towards monolayers of lipid. Its specific radioactivity was approx. 19.3 $\mu\text{C}/\mu\text{mole}$, indicating that about an average of 1.25 residues/molecule had been carboxymethylated. All of the cytochrome *c* used in the present experiments was in the oxidized form: in other experiments it was observed that chemical reduction of the protein affected its interaction with lipid films (P. J. Quinn & R. M. C. Dawson, unpublished work).

On electrophoresis in 15% polyacrylamide as described by Palmer & Dawson (1969) the carboxymethylated product had the same cathodic mobility as cytochrome *c* at pH 4.0 whereas at pH 5.9 the mobility was very slightly retarded. This is to be expected because the carboxylic acid groups introduced would be ionized at the higher pH. All the radioactivity in the gel was associated with the protein component.

Complete carboxymethylation of native cytochrome *c* was carried out at pH 3, a higher concentration of iodoacetate being used than was employed for the labelling procedure (Ando *et al.* 1966*a,b*).

Preparation of phosphatidylethanolamine. Egg phos-

phatidylethanolamine was prepared as described by Dawson (1963). T.l.c. indicated that the phospholipid was homogeneous. It existed primarily in the diacylated form (90%+), but successive chemical degradation (Dawson, Hemington & Davenport, 1962) indicated that some ethanolamine plasmalogen and glycerol ether phospholipid (ethanolamine) were also present. G.l.c. showed that the main fatty acids liberated by saponification were stearic acid (43%), oleic acid (27%), palmitic acid (13%), linoleic acid (8%) and docosahexaenoic acid (5%). The phospholipid was stored at -15° in chloroform. Immediately before spreading as a monolayer it was diluted with 4 vol. of light petroleum (b.p. 40–60 $^{\circ}$). Storage of the phospholipid in the presence of light petroleum was found inadvisable as it hastened autoxidation. Hydrogenation was carried out by the method of Farquhar, Insull, Rosen, Stoffel & Ahrens (1959) in chloroform solution. The product was purified at least twice by running the phospholipid as a band on silica gel H with chloroform-methanol-water-acetic acid (135:50:4:1, by vol.) as solvent and eluting with ethanol-chloroform-water (10:3:2, by vol.). G.l.c. of the fatty acids from the saponified phospholipid indicated that no unsaturated fatty acids remained; nearly 80% was stearic acid.

Monolayer techniques and apparatus. The basic apparatus (Dawson, 1969) consisted of a Perspex trough stirred by a reciprocating glass-sheathed bar activated magnetically from the underside at a constant rate of 16 strokes/min. The capacity of the trough was 75 ml.

Surface pressure was recorded continually by using a glass rod (0.3 mm. diam.) dipping into the surface film. The downward force on the rod was measured by an electronic micro-force balance (C.I. Electronic Ltd., Slough, Bucks.) and recorded continuously on a three-channel pen recorder (Rikadenki Kogyo Co. Ltd., Tokyo, Japan). It was found that the glass surface rapidly became contaminated and this was obviated when the dipping rod was coated with carbon from a benzene flame (Cheesman, 1946a,b).

The surface potential was measured with an americium air-ionizing electrode (Quarles & Dawson, 1969) and the potential output of the millivoltmeter fed into another channel of the pen recorder. It is usual to express the change of interfacial potential produced by spreading a phospholipid film at the air/water interface as ΔV , but for convenience throughout this investigation ΔV represents the change in surface potential brought about by allowing protein to react with the phosphatidylethanolamine film except where otherwise stated.

The surface radioactivity was measured with a flow counter constructed by Mr J. Bounden of this Institute. It consisted of a glass cylindrical chamber (6 cm. long $\times$ 4.5 cm. diam.) closed at one end with a window of 6 μ m.-thick polyethyleneterephthalate (Melinex; Imperial Chemical Industries Ltd., Welwyn Garden City, Herts.). This window was aluminized on the outside and the whole chamber, including the window, coated with colloidal carbon (Aquadag) and shielded from light by enclosure in a light-proof box. The central anode was an annulus of tungsten wire. With a gas flow of helium + butane (98.5:1.5) this gave a plateau of several hundred volts, the operating voltage being near 1600 v. The pulses of ionization current from the counter were amplified in an Ekco Ratemeter N 522 C and the output, integrated over 20 sec., was fed into the remaining channel of the pen recorder. The background of the

counter amounted to about 3 counts/sec. Interference from the americium electrode was considerably reduced by interposing a 2 mm. lead shield.

The relationship between surface radioactivity and the amount of protein was determined by spreading a film of 11 μ g. of 14 C-labelled cytochrome *c* in 70% ethanol on the surface of *m*-NaCl (80.1 cm.²) and measuring the surface radioactivity. The ratio between the radioactivity and molecules of cytochrome *c*/cm.² of surface remained constant on compressing the protein film to 49.5 cm.², suggesting that no protein had been lost to the subphase. Therefore the measurements of surface radioactivity could be used to calculate (mol.wt. of cytochrome *c* 12363) an approximate value for protein molecules/cm.² in the surface in subsequent experiments.

The whole of the trough, with counter, dipping plate and air-ionizing electrode, was enclosed in a Perspex box with removable front to protect it from draughts and dust. The pH of the subphase was adjusted and maintained when necessary by using a Radiometer pH-stat delivering NaOH or HCl solution to the subphase.

The trough was filled with water (80 ml.) that had been distilled over alkaline KMnO_4 ; the surface was then 'swept' with a Perspex barrier and an appropriate volume of the phosphatidylethanolamine solution (chloroform-light petroleum, 1:4, v/v) introduced on to the surface from an Agla micrometer syringe (Burroughs Wellcome and Co., Beckenham, Kent). After the solvent had evaporated, the film was adjusted to the desired pressure by compressing with the barrier. When the surface characteristics had remained constant for some minutes the protein was introduced into the bulk phase by injection through the film with a hypodermic syringe. This technique of interaction originally introduced by Doty & Schulman (1949) was found most favourable for the interaction of γ -globulin and cholesterol monolayers by Colacicco *et al.* (1967). After each run the trough was cleaned by copious washing with distilled water, and it was never allowed to dry out.

Particle microelectrophoresis. Liquid-paraffin emulsions were prepared by repeated rapid expulsion of paraffin and water from a syringe to give particles of approx. 10–20 μ m. diam. Phosphatidylethanolamine particles were prepared by shaking with water the solid phospholipid obtained by evaporating a chloroform solution. A sample of either suspension was added to a solution of the protein in 10 mM-NaCl at a concentration indicated in the legend to Fig. 13. After 20 min. this suspension (0.5 ml.) was diluted to 5 ml. with 10 mM-NaCl and adjusted to the required pH either with the pH-stat or by adding the appropriate buffer. The amount of initial suspension added to the protein solution was adjusted to give about five to ten particles per field of view, at the final dilution. The mobility of the particles was assessed at a temperature of 23 $^{\circ}$ in an apparatus similar to that described by Bangham, Flemans, Heard & Seaman (1958); the stationary layer was determined by using human erythrocytes. Afterwards the suspension was removed from the electrophoresis tube and, where no buffer had been added, the pH was immediately redetermined. If a small change in pH had occurred the mean of the original and final pH was taken. When the cytochrome *c* solution and liquid-paraffin suspensions were adjusted to the required pH before interaction the electrophoretic mobilities found were very similar to those obtained by adjusting the pH after the interaction.

Fig. 1. Interaction of cytochrome *c* and a phosphatidylethanolamine monolayer at 10 dynes/cm. initial pressure. ^{14}C -labelled cytochrome *c* ($34\text{ }\mu\text{g}$. labelled + $56\text{ }\mu\text{g}$. native) was added to the stirred subphase (75 ml., 10 mM-NaCl) at 0, 40 and 80 min. as indicated by the arrows. Continuous recordings were made of the changes in surface pressure ($\Delta\pi$, —), surface potential (ΔV ,), and surface radioactivity (ΔR , ----) and the results are plotted from the pen-recorder traces.

RESULTS

The changes with time of the surface pressure, potential and radioactivity produced by adding successive $90\text{ }\mu\text{g}$. portions of cytochrome *c* below a phosphatidylethanolamine film at 10 dynes/cm. are illustrated in Fig. 1. When the subphase was stirred there was an immediate increase in the surface pressure ($\Delta\pi$) and the surface potential (ΔV) became more positive.

This increase in π , which can be associated with physical penetration of at least part of the protein molecule into the film, reached a maximum of 18 dynes/cm. and further addition of cytochrome *c* caused no additional change. Changes in V occurred simultaneously with those in π , but the surface radioactivity (R) continued to increase after the change in surface pressure had ceased. Further movements in ΔR were observed for each addition of cytochrome *c*, indicating that protein continued to be concentrated in the surface phase even though penetration was complete. This phenomenon is referred to below as adsorption.

Cessation of stirring caused the penetration and adsorption to almost cease, suggesting that the rate of interaction was determined to some extent by the supply of protein coming to the surface. The absence of a point of inflexion in the ΔR curve when the change in surface pressure ceased indicates

that initially all the protein molecules arriving at the phospholipid monolayer penetrate into the film. Once this penetration is complete then the protein molecules arriving at the surface continue to accumulate at the interface by the process of adsorption.

Calculation from ΔR and the area per molecule of phosphatidylethanolamine in the monolayer indicated that at this film pressure the penetrated protein occupied an area equal to 22 phospholipid molecules ($2130\text{ }\text{\AA}^2$). About 8% of the protein added to the subphase was now associated with the film. Heating the cytochrome *c* at 65° for 10 min. before it was added to the subphase produced no significant difference in the interaction, although the first step of thermal denaturation is known to occur at 53° (Myer, 1968). However, it is known that this first step of thermal denaturation is freely reversible unless the time of heating is prolonged (Margoliash & Schejter, 1966).

When increasing amounts of cytochrome *c* were added to the subphase below phosphatidylethanolamine films at initial pressures of 10 and 2 dynes/cm., the surface pressure and potential increments were greater with the lower-pressure films (Fig. 2). This is consistent with the expectation that the less-dense packing of the phospholipid molecules at the lower pressure would facilitate penetration of the protein molecules. However, there were no inflexion

Fig. 2. Changes in surface pressure and potential on addition of cytochrome *c* below monolayers of phosphatidylethanolamine. Increasing amounts of native cytochrome *c* were added below phosphatidylethanolamine films at 2 dynes/cm. (●) and 10 dynes/cm. (○) initial pressures. —, Surface pressure (π); ····, change in surface potential (ΔV); subphase 10 mM-NaCl (75 ml.).

points in the increase of π on adding protein as have been observed by other workers for the interaction of various proteins and polypeptide hormones with low-pressure (2 dynes/cm.) monolayers of lipids other than phospholipids, e.g. stearic acid, cholesterol, dihydroceramide lactoside (Eley & Hedge, 1956, 1957; Colacicco *et al.* 1967; Snart & Sanyal, 1968). We have also observed that a similar inflexion point is present in the curve relating $\Delta\pi$ and cytochrome *c* added below a phosphatidylcholine monolayer.

It appeared that the penetrated cytochrome *c* was not in true equilibrium with that in the bulk phase, since on sliding a film of phosphatidylethanolamine that had been penetrated by cytochrome *c* on to a fresh subphase containing no cytochrome *c* there was no loss of pressure on maintaining the area constant, and the same result was obtained when an attempt was made to remove cytochrome *c* by perfusion of the subphase.

The decrease in $\Delta\pi$ with initial starting pressures of the phosphatidylethanolamine films shown in Fig. 2 is more systematically examined in Figs. 3 and 4. Fig. 3 shows that $\Delta\pi$ and ΔV decreased linearly as the initial starting pressure of the monolayers increased and that with film pressures above 24 dynes/cm. there was no penetration. This pattern is similar to that observed for the interaction of γ -globulin with dihydroceramide lactoside monolayers (Colacicco *et al.* 1967) and of the apoprotein of high-density plasma lipoprotein with phosphatidylcholine (Camejo, Colacicco & Rapport, 1968). ΔR_p , the values of ΔR at the time when penetration just ceased, were related to the initial pressure in the same way, but the final equilibrium values (ΔR_E) indicated that appreciable adsorption had occurred even in the absence of penetration. Whereas the

Fig. 3. Effect of initial starting pressure of phosphatidylethanolamine monolayers on the penetration characteristics of cytochrome *c*. A 90 μ g. portion of 14 C-labelled cytochrome *c* (34 μ g. labelled + 56 μ g. native) was added to the subphase (10 mM-NaCl); ○—○, Increase in surface pressure, $\Delta\pi$; ●·····●, change in surface potential, ΔV ; △—△, change in surface radioactivity (ΔR) when $\Delta\pi$ became constant; ▲—▲, final value of ΔR reached.

Fig. 4. Penetration of native and carboxymethylated cytochrome *c* into monolayers of egg phosphatidylethanolamine (—) and hydrogenated egg phosphatidylethanolamine (---) at various pressures. Subphase of 10 mM-NaCl (75 ml.). ○, Native cytochrome *c*; △, 14 C-labelled cytochrome *c*; ●, fully carboxymethylated cytochrome *c*.

ΔR_p component (penetration) decreased as the pressures of the monolayers were increased the $\Delta R_E - \Delta R_p$ component (adsorption) increased.

The complete hydrogenation of the egg phosphatidylethanolamine caused a difference in the slope of the penetration ($\Delta\pi$) plotted against the initial starting pressures of the monolayers, but the minimum initial pressure at which no penetration occurred remained the same at approx. 24 dynes/cm. (Fig. 4). In Fig. 5 the surface potentials and pressures of native and hydrogenated phosphatidylethanolamine are compared with the area per

Fig. 5. Area occupied by each phosphatidylethanolamine molecule in a monolayer plotted against surface potential and pressure. —, Surface pressure, π ; ····, surface potential representing the change in interfacial potential of a pure air/water interface occurring when the phosphatidylethanolamine is spread as a monolayer. A, Hydrogenated egg phosphatidylethanolamine; B, egg phosphatidylethanolamine.

molecule occupied by the phospholipid molecules at the interface. The penetration into phosphatidylethanolamine monolayers at all pressures was not influenced by partial or complete carboxymethylation of the cytochrome *c* molecule (Fig. 4).

When cytochrome *c* was allowed to penetrate into a phosphatidylethanolamine monolayer at low pressure on a subphase of 10 mM-sodium chloride and the monolayer compressed to 40 dynes/cm., only a small amount of the penetrated protein left the film (Fig. 6). Thus on suddenly re-expanding the film to the same area as it originally occupied the pressure fell to a value appreciably above that of the original film before interaction with the protein. The amount of protein removed on compression to 40 dynes/cm. was independent of the time allowed for the penetration to occur. The surface potential changes, ΔV , also indicated that protein was still associated with the film after compression. Thus on compression there was an immediate increase of ΔV corresponding to the greater surface density of phospholipid molecules (Fig. 5). As the film was expanded to its original area, ΔV fell to a value only slightly below the maximum reached during the penetration. The results are therefore consistent with the compression squeezing out a certain proportion of protein

Fig. 6. Ejection of penetrated cytochrome *c* from a monolayer of phosphatidylethanolamine by compression. A 90 μ g. portion of ^{14}C -labelled cytochrome *c* was added at zero time; —, surface pressure; ····, surface potential (difference between interfacial potential of an air/water interface with and without a phosphatidylethanolamine monolayer). (a) Subphase of 1 M-NaCl (75 ml.); (b) subphase of 10 mM-NaCl (75 ml.).

Fig. 7. Adsorption of ^{14}C -labelled cytochrome *c* on a phosphatidylethanolamine monolayer at 29 dynes/cm. ΔR , maximum increase in surface radioactivity on adding successive increments of cytochrome *c* (34 μg . labelled + 56 μg . native) to the subphase (75 ml., 10 mM-NaCl); 40 min. was allowed for equilibrium after each addition.

Fig. 8. Effect of NaCl on the interaction of cytochrome *c* with a phosphatidylethanolamine monolayer at 10 dynes/cm. —, Change in surface pressure, $\Delta\pi$; ----, change in surface radioactivity, ΔR . Curves A and C, subphase 10 mM-NaCl, then solid NaCl added at 35 min. to make final concentration 1 M. Curves B and D, subphase 1 M-NaCl.

molecules (or parts of all protein molecules) from the monolayer, but there is no evidence that protein molecules leave the surface and return to the bulk phase. In fact observation on the surface radioactivity suggested that all the protein molecules remained in the surface phase on compression.

Adsorption on high-pressure monolayers. Adsorption of ^{14}C -labelled cytochrome *c* still occurred on to a film of phosphatidylethanolamine at 29 dynes/cm., a pressure too high for penetration to occur, and consequently no changes in $\Delta\pi$ and ΔV were shown. A linear relationship of ΔR with concentration occurred initially, with saturation at approx. 1 mg. of cytochrome *c* in the subphase (Fig. 7). In the penetration experiments with films at 10 dynes/cm., the maximum change in $\Delta\pi$ occurred with less

than ten times this concentration (Figs. 1 and 2). At maximum adsorption the ratio of phosphatidylethanolamine molecules in the film to cytochrome *c* molecules bound is approx. 4:1. A Langmuir-type plot of the ratio between cytochrome *c* concentration and ΔR against cytochrome *c* concentration was linear up to 0.5 mg. of the protein in the subphase. Although a Langmuir relationship suggests reversibility, the adsorption did not appear to be so since on perfusing the subphase and removing the bulk-phase cytochrome *c* no surface radioactivity was lost.

Effect of sodium chloride concentration on penetration and adsorption. Since the reaction between cytochrome *c* and phosphatidylethanolamine has been considered to be primarily an electrostatic type of interaction (see e.g. Green & Fleischer, 1963), the effect of increasing the content of sodium chloride in the subphase was examined. Raising the concentration to 1 M did not prevent penetration, but the time to reach equilibrium in $\Delta\pi$ was appreciably greater (Fig. 8).

On 0.01 M-sodium chloride compression caused only a small proportion of the penetrated protein to be ejected, but on 1 M-sodium chloride all of the penetrated component was desorbed on compression to 40 dynes/cm. (Fig. 6). In addition the major part of protein already taken up from a subphase of 10 mM-sodium chloride could be removed by compression if salt was added to bring the concentration to molar. When the protein was allowed to penetrate the film on a subphase of 10 mM-sodium chloride and the concentration then increased to 1 M-sodium chloride none of the penetrated protein was removed (no change in $\Delta\pi$). However, the surface radioactivity clearly showed that protein was leaving the surface phase (Fig. 8). This suggested that the higher sodium chloride concentration was displacing adsorbed cytochrome *c* and not affecting the penetrated protein.

To examine the effect of sodium chloride on adsorption uncomplicated by penetration, the reaction of ^{14}C -labelled cytochrome *c* with phosphatidylethanolamine monolayers of 25 dynes/cm. was examined. Conducting the interaction in M-sodium chloride very considerably reduced the increase in surface radioactivity (Fig. 9) obtained on adding the protein. Moreover, if cytochrome *c* was adsorbed on the film on 10 mM-sodium chloride, increasing the concentration to M-sodium chloride displaced a proportion of the protein associated with the monolayer (Fig. 9). The amount of adsorbed protein removed decreased with the time of the interaction until after about 40 min. no further loss was observed (Fig. 10). The amount removed appeared to be largely independent of the starting pressure of the film in the range 25–40 dynes/cm.

Effect of subphase pH on adsorption. In these

Fig. 9. Adsorption of ^{14}C -labelled cytochrome *c* on phosphatidylethanolamine monolayers at 25 dynes/cm. in 10 mM- and 1 M-NaCl. A 90 μg . portion of cytochrome *c* (34 μg . labelled + 56 μg . native) was added to the subphase consisting of 10 mM-NaCl (upper curve) and 1 M-NaCl (lower curve). After 35 min. solid NaCl was added to the 10 mM-NaCl subphase to bring the final concentration to 1 M.

Fig. 10. Removal of cytochrome *c* adsorbed on phosphatidylethanolamine monolayers by adding NaCl. Monolayers of phosphatidylethanolamine at various pressures ($\circ$, 25 dynes/cm.; $\bullet$, 35 dynes/cm.; Δ , 40 dynes/cm.) on 10 mM-NaCl (75 ml.) were allowed to interact with ^{14}C -labelled cytochrome *c* and solid NaCl was added after various times to bring the subphase concentration to 1 M.

experiments separate monolayers were used for each subphase pH examined. With films at 26 dynes/cm. no penetration of the protein was indicated by a surface pressure increment ($\Delta\pi$) at any pH and the surface potential became more positive on adding the protein at all pH values below 9 (Fig. 11). The ΔV increased as the subphase pH was lowered to 3 with a short plateau between pH 4 and 6. The surface radioactivity indicated that ^{14}C -labelled cytochrome *c* adsorption to the monolayer increased steadily from pH 3 to 8.5 and then fell precipitously at higher pH values (Fig. 11).

Effect of subphase pH on penetration. An initial pressure of 10 dynes/cm. was used with a subphase of M-sodium chloride to prevent adsorption. In this system the initial rate of the interaction (6 min. values) showed (both with ΔR and $\Delta\pi$) a steadily decreasing penetration of protein between pH 3.5 and 7.5, a plateau between 7.5 and 9.5 and then a sharp fall in the more alkaline region (Fig. 12). This would be expected from the difference in the electrostatic charge on the protein and the phosphatidylethanolamine film as judged by microelectrophoresis (see below) (Fig. 13), if the initial interaction during penetration were primarily electrostatic in nature. Between pH 3 and 6 the final penetration values of $\Delta\pi$ were very similar to those at 6 min., but at higher pH values this relationship was lost (Fig. 12). In spite of a much lower rate of penetration the final $\Delta\pi$ showed a peak, reaching a maximum at pH 9, that was not accompanied by an increase in the surface radioactivity. The protein

Fig. 11. Effect of subphase pH on the adsorption of ^{14}C -labelled cytochrome *c* on phosphatidylethanolamine films at 26 dynes/cm. The phosphatidylethanolamine films were spread on a subphase of 10 mM-NaCl. Each pH value was maintained with a pH-stat and 90 μg . of ^{14}C -labelled cytochrome *c* (34 μg . labelled + 56 μg . native) added. $\circ$ — $\circ$, Change in surface pressure, $\Delta\pi$; $\circ$... $\circ$, change in surface potential, ΔV ; $\circ$ — $\circ$, change in surface radioactivity, ΔR .

was therefore penetrating in a different way, producing a far larger surface-pressure increment per molecule.

Electrophoresis of lipid particles in the presence and absence of proteins at various pH values. The electrophoretic mobility of phosphatidylethanolamine particles suspended in 10 mM-sodium chloride showed that the particles were isoelectric at pH 3

Fig. 12. Effect of subphase pH on the reaction between ^{14}C -labelled cytochrome *c* and phosphatidylethanolamine monolayers at 10 dynes/cm. ^{14}C -labelled cytochrome *c* (34 μg . labelled + 56 μg . native) was introduced into the subphase (75 ml. of M-NaCl), the pH being maintained with a pH-stat. (a) $\bullet$ — $\bullet$, Change in surface pressure ($\Delta\pi$); $\circ$ — $\circ$, change in surface radioactivity (ΔR) after 6 min. (b) $\Delta\pi$ and ΔR on completion of the protein-phospholipid interaction.

and had a steadily increasing anodic mobility up to pH 10.5 (Fig. 13). A similar pH-mobility curve for this phospholipid has been reported by Bangham, Pethica & Seaman (1958). In the presence of an excess of cytochrome *c* the phosphatidylethanolamine particles showed zero mobility at pH 6.8.

The pH-mobility relationships of cytochrome *c* (both native and carboxymethylated) adsorbed on liquid-paraffin particles were also measured. There was no significant difference between the carboxymethylated and native forms, but both proteins caused the liquid-paraffin particles to become isoelectric at pH 8.8, some 1.2 pH units below the isoelectric point as measured by conventional methods with non-combining buffers (Barlow & Margoliash, 1966). Similar experiments performed with other proteins having both low (serum albumin) and high isoelectric points (ribonuclease A, clupeine) gave isoelectric points at pH values very close to the accepted isoionic points.

Fig. 13. Electrophoretic mobilities of phosphatidylethanolamine and liquid-paraffin particles, that had interacted with various proteins, as a function of pH. Unless otherwise stated the mobilities were measured in 10 mM-NaCl adjusted to the requisite pH with a pH-stat. A, Phosphatidylethanolamine particles. B, Liquid-paraffin particles; bovine serum albumin (4 mg./ml.) in 0.05 M-acetic acid-sodium acetate buffers. C, Phosphatidylethanolamine particles; native cytochrome *c* (4 mg./ml.). D, Liquid-paraffin particles; native cytochrome *c* ($\blacktriangle$, 2 mg./ml.; $\circ$, 4 mg./ml.; $\square$, 8 mg./ml.); carboxymethylated cytochrome *c* ($\bullet$, 4 mg./ml., $\blacksquare$, 4 mg./ml. in 10 mM-NaOH-glycine buffers). E, Liquid-paraffin particles; ribonuclease A (4 mg./ml.). F, Liquid-paraffin particles; clupeine (4 mg./ml.) in 50 mM-KCl-NaOH buffers.

DISCUSSION

The measurement of surface radioactivity introduces a new physicochemical parameter in the study of interactions between protein and lipids orientated as monolayers at the air/water interface. An increase in the surface radioactivity shows that labelled protein has moved into the surface phase, but it does not indicate how the protein is orientated in relation to the lipid molecules.

Carboxymethylation of the protein did not affect its interaction with the phosphatidylethanolamine films although the treated protein was less positively charged at pH values above 5 and showed a slightly different migration on polyacrylamide-gel electrophoresis. It seems likely that the addition of one or two free carboxylic acid groups to the 13 already present in the protein did not affect the basic interaction. The degree of carboxymethylation of preparations used in most experiments did not affect the biological activity as measured by the procedure of Horecker & Kornberg (1946), nor was the

isoelectric point of liquid-paraffin particles that possessed an adsorbed layer of cytochrome *c* significantly affected by prior carboxymethylation of the protein. It was, of course, not possible to assess adsorption without penetration on to phosphatidylethanolamine monolayers in the absence of carboxymethylation. Mirsky & George (1967*a*) have shown that monocarboxymethylated cytochrome *c* has an optical-rotatory-dispersion spectrum almost identical with that of native cytochrome *c*. This indicates that a degree of carboxymethylation equivalent to that used in the present labelling of the protein has little effect on its basic conformation. However, carboxymethylation does have a small but measurable effect on the penetration of the protein into phosphatidylcholine films (P. J. Quinn & R. M. C. Dawson, unpublished work).

The additional measurement of surface radioactivity has brought out several facts concerning the interaction that cannot be appreciated from surface-pressure and potential measurements. First, binding of the protein to the lipid film can occur in the complete absence of any change in the surface pressure or potential, and is referred to in this paper as adsorption. Secondly, although the concentration of protein in the subphase is linearly related to the protein bound to the film (both penetrated and adsorbed) until the latter is almost saturated, this does not necessarily imply that all the protein added has interacted with the film. In fact, in the present experiments only a small proportion of the added cytochrome *c* was actually associated with the phospholipid film. This completeness of the interaction between proteins and lipids has often been assumed with differing proteins and peptide hormones and has led to the calculation of stoichiometric relationships between the interactants, and to the suggestion that excess of protein is bound in the form of an underlying multilayer. Although such conclusions are not necessarily wrong with other proteins, the present experiments with ¹⁴C-labelled cytochrome *c* illustrate that caution is necessary in the interpretation of such results. The small proportion of the added cytochrome *c* that became bonded to the phosphatidylethanolamine monolayer was clearly not in equilibrium with that remaining in the subphase. Thus no loss of the attached protein was observed when the cytochrome *c* remaining in the subphase was removed. This tight irreversible bonding is probably a consequence of the multipoint attachment that is to be expected for such a large macromolecule on a charged indented surface. There is no evidence that such a tight bonding produces denaturation of the protein, a point that is amplified below.

With such an irreversible bonding it is difficult to think of the lipid surface under a given set of conditions containing a certain number of acceptor

sites for the protein. Thus it would be expected that such sites would be filled, provided that protein still remained in the subphase and collisions with the lipid film were still occurring. The concentration of protein would affect the rate of bonding but not the final amount of attachment. Why in the present instance both penetration and adsorption are dependent on protein concentration with a fixed proportion of the protein being bonded and remaining in the subphase is not clear.

With cytochrome *c* and phosphatidylethanolamine monolayers there was no evidence for any stoichiometric relationship, as for example one amino acid residue under each orientated phospholipid head group in the monolayer. In fact, calculation shows that the relationship between the two continuously changed when the pressure of the film was varied and the pH of the subphase altered. Certain loosely defined complexes have been described on the interaction of aqueous suspensions of phosphatidylethanolamine particles and cytochrome *c*, and these vary in composition with the degree of saturation of the phosphatidylethanolamine fatty acid chains, the pH and ionic strength of the reaction medium (Reich & Waino, 1961; Das *et al.* 1964, 1965; Shipley, Leslie & Chapman, 1969).

Penetration of cytochrome c into phosphatidylethanolamine monolayers at pressures below 24 dynes/cm. The addition of cytochrome *c* to the subphase often resulted in an increase in pressure of the phospholipid film to well above that of the collapse pressure of the protein itself at the air/water interface. Measurements of force-area relationships showed that the collapse pressure of cytochrome *c* films varied between 10 and 16 dynes/cm. as the pH of the subphase was varied, reaching a maximum at pH 9, in general agreement with the results of Harkins & Anderson (1938), who used an impure cytochrome *c* preparation (0.34% iron). This implies that there is a substantial interaction between the phospholipid and cytochrome *c* at low pressures. The interaction (penetration) appears to be initially electrostatic in nature. Thus the rate of penetration was slowed by the addition of 1M-sodium chloride to the subphase, although even in this instance, when electrostatic bonding had been weakened, the final extent of the penetration appeared to vary predictably with pH according to the relative electrostatic nature of the interactants as judged by the microelectrophoretic properties of pure phosphatidylethanolamine and the titration curve of the protein (Paul, 1951). Although changes in ionic strength and pH can cause conformation changes in cytochrome *c* (Myer, Murphy & Harbury, 1966; Myer, 1968) that could affect the penetration, it seems more likely that the initial reaction is dependent on the net charge on the

protein/water interface relative to that of the electrostatic conditions at the phospholipid/water interface. Salem (1962) has indicated that other forces apart from ionic interactions between protein and lipids are not of significance when the participants are separated by more than about 60 Å. A similar dependence on the relationship between the electrical state of haemoglobin, albumin, insulin and γ -globulin and their penetration into positively and negatively charged monolayers of amphiphiles was indicated by the work of Matalon & Schulman (1949), Eley & Hedge (1957) and Baranova, Zhukova & Deborin (1965).

The final result of the electrostatic interaction is that the protein penetrates between the orientated molecules of phospholipid in the film, thus causing the increase in surface pressure. There is no absolute evidence for whether at very low pressures this penetration represents a 'solution' of whole protein molecules in the film to form what is tantamount to a mixed film of protein and phospholipid or a limited penetration of the hydrophobic side chains of the amino acid residues, of which cytochrome *c* has about eight main regions (Margoliash & Schejter, 1966). Calculation of the space occupied by the penetrated protein molecules in a film at 10 dynes/cm. from the amount of pressure increase and the force-area curves of the phospholipid suggests that this was well below the collapse area of a protein molecule at an air/water interface. Our results therefore tend to support the suggestion by Eley (1957) that protein molecules lie under very expanded monolayers of lipid rather than being physically 'dissolved' in the film (Matalon & Schulman, 1949).

Once the penetration was achieved on a 10 mM-sodium chloride subphase ejection occurred only to a limited degree on compressing the film. It has been suggested that such limited loss of protein on compressing a film is due to irreversible denaturation of the 'solubilized' protein that occurs at the air/water interface (Matalon & Schulman, 1949). In the present instance this seems unlikely, because irreversible conformation changes in cytochrome *c* are produced only under extreme conditions and the unfolding of the protein produced by such agents as urea or heat is largely reversible (Myer, 1968). In fact when cytochrome *c* is recovered after its interaction with particulate phosphatidylethanolamine it still possesses its original biological, spectroscopic and electrophoretic properties (Reich & Waino, 1961). Such unfolding that must undoubtedly occur in the reaction (Hardesty & Mitchell, 1963) is therefore likely to be reversible. Complete ejection on compression can be obtained if the penetration of the cytochrome *c* has occurred in M-sodium chloride or if the subphase is made M-sodium chloride after penetration, which suggests

that the forces holding the protein into the film on compression are mainly or wholly electrostatic in nature.

The finding that the limiting pressures at which penetration ceases are identical (24 dynes/cm.) for both native and hydrogenated phosphatidylethanolamine is somewhat surprising. Calculation of the available 'space' for penetration from the areas per molecule occupied at 24 dynes/cm. and collapse (Fig. 5) indicates that far more is available in the unsaturated film, which suggests that the extent of penetration is related to the thermodynamic work done on entering the monolayer. Shah & Schulman (1967) have obtained evidence that the phospholipase A hydrolysis of phosphatidylcholine monolayers ceased at a limiting pressure, which increased as the saturation of the phospholipid was decreased. If the hydrolysis of the monolayer coincides with protein penetration this is contrary to the present results with a different protein. The observation that at film pressures below 24 dynes/cm. cytochrome *c* penetrates more readily into unsaturated phosphatidylethanolamine monolayers could indicate that if penetration can begin the interaction is assisted by some reaction between the unsaturated bonds and protein. Das *et al.* (1964) found that complex-formation between cytochrome *c* and phosphatidylethanolamine particles in suspension occurred more readily when the latter were unsaturated, but this could have been due to the greater ease of dispersion of the unsaturated phospholipid, which would have presented a larger surface area for reaction.

Adsorption of cytochrome c on phosphatidylethanolamine monolayers. The adsorption without penetration of cytochrome *c* on monolayers of phosphatidylethanolamine at all pressures revealed by the surface-radioactivity measurements again appears to be initially electrostatic in nature. Thus the interaction was blocked by adding M-sodium chloride to the subphase, a manoeuvre that would diminish the intensity of the electrostatic field around each reactant. In fact after a small initial interaction in the presence of M-sodium chloride no further adsorption occurred, in contrast with penetration of the protein where M-sodium chloride merely slowed the reaction rather than preventing it completely. It is clear that once electrostatic interaction has occurred the adsorption is subsequently stabilized by non-ionic bonding, since initially the adsorbed protein can be substantially displaced by M-sodium chloride but later only a small proportion can be desorbed. The nature of this second interaction is unknown, but, since it causes no increment in the surface pressure, a direct non-ionic interaction without penetration between the protein and the polar head group of the phospholipid is indicated. This could be for example through

van der Waals bonding between protein side chains and the methylene groups of the ethanolamine orientated coplanar to the surface or through hydrogen bonding.

Effect of subphase pH on adsorption and penetration. The effect of maintaining the subphase pH on the adsorption of cytochrome *c* can be substantially explained by assuming that the reaction is mainly electrostatic in nature. The interpretation of the results is, however, somewhat complicated since the electrostatic field adjacent to the monolayer would cause an adsorption of protons on the negatively charged monolayer. With a high-pressure film of phosphatidylethanolamine the final surface radioactivity reached (and also the rate of reaction) is consistent with the relative electrostatic states of the protein and phospholipid. This difference can be appreciated from the evidence obtained on the relative electrophoretic mobilities of phosphatidylethanolamine and the protein adsorbed on liquid-paraffin particles (Fig. 13). As the pH is increased to above pH 8.5 there is a dramatic decrease in the adsorption on the monolayer, this presumably being due to the depolarization of the ϵ -amino groups of the 17 lysine residues in the protein. This is illustrated by the shape of the titration curve of cytochrome *c* as described by Paul (1951). The electrophoretic data of the protein adsorbed on liquid-paraffin particles indicate that the isoelectric point is well below that of the true isoelectric point of the protein in free solution (10.05; Theorell & Åkesson, 1941*a,b,c*). We interpret this to mean that the protein-protein interactions of cytochrome *c* are in this instance relatively weak compared with the other proteins examined (clupeine, ribonuclease A, serum albumin). It is generally believed that such proteins build up multilayers over the liquid-paraffin particles; each layer shows a smaller degree of unfolding so that eventually the outer layer of protein has a conformation that is scarcely different from the protein in free solution (Abramson, 1932). With cytochrome *c*, however, it is clear that multilayer formation is not sufficient to suppress the anodic mobility of the liquid-paraffin particles, so the whole pH-mobility curve is shifted towards a more acid pH. If phosphatidylethanolamine particles are used that originally have a greater anodic mobility than liquid-paraffin particles the pH-mobility curve is shifted even further towards an acid pH. The absence of the build-up of multilayers of cytochrome *c* on phosphatidylethanolamine is also indicated by the adsorption of the protein on high-pressure monolayers (Fig. 7). Calculation shows that, at 'saturating' concentrations, the area occupied by each protein molecule was about 220 Å². This is equivalent to about half the area per molecule of protein at collapse pressure. Polymeric

forms of cytochrome *c* can be formed when the conformation of the protein is altered sufficiently to allow exposed groups to react with other cytochrome *c* molecules (Margoliash & Lustgarten, 1962). Presumably in the present instance the electrostatic deformation of the first layer of adsorbed protein is only sufficient to allow limited protein-protein interactions and successive multilayers are not formed.

When cytochrome *c* was adsorbed on a phosphatidylethanolamine monolayer and the subphase pH maintained with a pH-stat, the change in surface potential increased below pH 9, presumably as the protein developed a greater net positive charge. This charge would influence the ionic dipoles on the phospholipid head groups, which, together with the intrinsic dipoles of the molecules and those of orientated water molecules, constitute the interfacial potential (Davies & Rideal, 1961). In the unbuffered system the absence of an appreciable change of surface potential on adsorption suggests that the surface protein has, at the most, only a small net positive charge.

The initial rate of penetration of cytochrome *c* into a phosphatidylethanolamine monolayer (10 dynes/cm., subphase M-sodium chloride) again appears to depend broadly on the relative differences in the electrostatic charges on protein and phospholipid (Fig. 11). This is in spite of the presence of M-sodium chloride, which would decrease the extent of the electrostatic field surrounding both reactants. As mentioned above this has the effect of lowering the rate of penetration although not preventing it. However, in contrast with the adsorption the electrostatic conditions of penetration seem to be more specific than those for adsorption. This is presumably a reflection of the much closer and more specific reaction of the participants in penetration. At pH values below 3 the phosphatidylethanolamine will acquire a net positive charge and repulsion of the highly positively charged protein occurs with a lower rate of penetration. As the phosphatidylethanolamine acquires a negative charge (Fig. 13) the rate of reaction becomes maximal. At this point the protein has a maximum positive charge and all its free carboxyl groups (10 glutamic acid, 3 aspartic acid) will be substantially discharged. As the pH increases these carboxyl groups become charged between 3.5 and 6 and the rate of adsorption falls in spite of the fact that the negative charge on the phospholipid is increasing. This suggests that the extent and nature of the charge on the protein may be of more importance in determining penetration than the net difference between the charges on the two reactants. Between pH 7 and 9.5 the rate of interaction does not change appreciably and this corresponds to a region where there are few ioniza-

tion changes on the surface of protein (Paul, 1951). Once the lysine ϵ -amino groups of protein become depolarized the rate falls rapidly and finally no penetration occurs at approx. pH 10.5–11, i.e. when both phospholipid and protein possess net negative surface charges.

The final extent of the penetration (Fig. 12) below 6 and above pH 9.5 follows a similar pattern to that of the initial rate of reaction. However, between pH 6.5 and 9.5 this is not so. At pH 6.5 the final extent of the penetration begins to increase as judged by the surface-pressure increment yet the number of protein molecules associated with the film as measured by the radioactivity actually falls. This suggests that in this pH region conformation changes occur in the protein that allow more parts of the molecule to penetrate into the film. It is surprising that this corresponds to a pH region where it is to be expected that little change would occur in the ionization of the protein moiety of the cytochrome *c*. However, studies of the optical-rotatory-dispersion spectra of horse heart cytochrome *c* have shown changes between pH 7 and 9.5 corresponding to the disappearance of the 695 nm. optical absorption band (Mirsky & George 1966, 1967b) and this transition, which is presumably related to changes of conformation round the haem region, may be connected with the penetration of the protein molecule into monolayers in this pH region.

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