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Interactions of Cytochrome *c* and [^{14}C]Carboxymethylated Cytochrome *c* with Monolayers of Phosphatidylcholine, Phosphatidic Acid and Cardiolipin

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1. The interactions between cytochrome *c* (native and [^{14}C]carboxymethylated) and monolayers of phosphatidylcholine, phosphatidic acid and cardiolipin at the air/water interface was investigated by measurements of surface radioactivity, pressure and potential. 2. On a subphase of 10mM-or M-sodium chloride, penetration of cytochrome *c* into egg phosphatidylcholine monolayers, as measured by an increase of surface pressure, and the number of molecules penetrating, as judged by surface radioactivity, were inversely proportional to the initial pressure of the monolayer and became zero at 20dynes/cm. The constant of proportionality was increased when the cytochrome *c* was carboxymethylated or decreased when the phospholipid was hydrogenated, but the cut-off point remained at 20dynes/cm. 3. Penetrated cytochrome *c* could be removed almost entirely by compression of the phosphatidylcholine monolayer above 20dynes/cm. 4. With phosphatidic acid and cardiolipin monolayers on 10mM-sodium chloride the binding of cytochrome *c* was much stronger and cytochrome *c* penetrated into films nearing the collapse pressure (> 40dynes/cm.). The penetration was partly electrostatically facilitated, since it was decreased by carrying out the reaction on a subphase of M-sodium chloride, and the relationship between the surface pressure increment and the initial film pressure moved nearer to that observed with phosphatidylcholine. 5. Surface radioactivity determinations showed that [^{14}C]carboxymethylated cytochrome *c* was still adsorbed on phosphatidic acid and cardiolipin monolayers after the cessation of penetration. This adsorption was primarily electrostatic in nature because it could be prevented and substantially reversed by adding M-sodium chloride to the subphase and there was no similar adsorption on phosphatidylcholine films. 6. The penetration into and adsorption on the three phospholipid monolayers was examined as a function of the pH of the subphase and compared with the state of ionization of both the phospholipid and the protein, and the area occupied by the latter at an air/water interface. 7. It is concluded that the binding of cytochrome *c* to phospholipids can only be partially understood by a consideration of the ionic interaction between the components and that subtle conformational changes in the protein must affect the magnitude and stability of the complex. 8. If cytochrome *c* is associated with a phospholipid in mitochondria then cardiolipin would fulfil the characteristics of the binding most adequately.

Phospholipids have been considered as the site of binding of cytochrome *c* to mitochondrial membranes and consequently there have been a number of studies in which the interaction between the isolated protein and pure phospholipid has been studied by a variety of physical techniques (Shipley, Leslie & Chapman, 1969*a*,*b*). Recently we investigated the same interaction by using monolayers of phosphatidylethanolamine at the air/water interface and [^{14}C]carboxymethylated cytochrome *c* introduced into the subphase (Quinn & Dawson, 1969). The advantage of using a monolayer technique is that the orientation of the molecules of phospholipid at the interface is precisely understood and the area occupied by each molecule can be determined over wide limits by careful expansion or compression of the film. We concluded that two types of interaction could occur, one involving a penetration of at least part of the cytochrome *c* molecule into expanded monolayers of phosphatidylethanolamine, and

another where the cytochrome *c* molecules are adsorbed on closely packed lipid molecules at the interface without penetrating. In the present investigations these studies have been extended to three other phospholipids that occur in mitochondria, namely phosphatidylcholine, cardiolipin and phosphatidic acid, and the characteristics of the interaction with cytochrome *c* determined.

EXPERIMENTAL

[^{14}C]*Carboxymethylation of cytochrome* c. The labelling of cytochrome *c* by reaction with iodo[2-^{14}C]acetic acid has been described by Quinn & Dawson (1969). The specific radioactivities of the preparations compared with those of the original iodoacetate indicated that 0·73–1·25 amino acid residues/cytochrome *c* molecule had been carboxymethylated. Like the native cytochrome *c* the labelled protein was always in the oxidized form. For convenience the product is referred to as '[^{14}C]cytochrome *c*'.

Preparation of phospholipids. Egg phosphatidylcholine was isolated as described by Dawson (1963) and phosphatidic acid was prepared from this by using the phospholipase D contained in pea seeds (Palmer & Dawson, 1969; Quarles & Dawson, 1969). 1,2-Dipalmitoylglycerylphosphorylcholine (kindly supplied by the late Dr T. Malkin) was purified by preparative t.l.c. on silicic acid.

Cardiolipin was prepared from ox heart ventricle. The lipids were extracted by homogenizing the tissues with chloroform–methanol (2:1, v/v), 2l./100g. of tissue for 60min. The filtered extract was washed with 0·154M-NaCl and equilibrated several times with upper phase to remove non-lipid impurities (Folch, Lees & Sloane-Stanley, 1957) and after evaporation of the extract in a rotary drier the dried residue was dissolved in chloroform and fractionated on a silicic acid (Mallinckrodt) column (140g., 6·0cm. diam.). Elution with methanol–chloroform (1:39, v/v) (200ml.) brought out some non-phospholipid material, methanol–chloroform (1:19, v/v) (300ml.) eluted most of the cardiolipin with other acidic lipids and a green pigment, and methanol–chloroform (1:9, v/v) (400ml.) some cardiolipin together with ethanolamine phosphoglycerides. The cardiolipin eluted by methanol–chloroform (1:19, v/v) was purified by preparative t.l.c. [silica gel H (0·25mm. thick), loading 20–25μg. of P/cm., solvent chloroform–methanol–water–aq. NH_3 (sp.gr. 0·88) (108:50:5:2·85, by vol.), saturation chamber at 4°]. The cardiolipin band (R_F 0·51) was located by exposing marker strips on either side to I_2 vapour and scraping off the silicic acid. The phospholipid was eluted with ethanol–chloroform–water (10:3:2, by vol.), the extract evaporated to dryness and the cardiolipin extracted into chloroform. It was homogeneous on t.l.c. (silicic acid) in a number of solvents and gave only bis(glycerylphosphoryl)glycerol on deacylation (Dawson, Hemington & Davenport, 1962).

The fatty acid composition of all three phospholipids determined by g.l.c. on polyethylene glycol adipate columns at 190° is given in Table 1.

Hydrogenation of phospholipids. Hydrogenation of phosphatidylcholine and cardiolipin in chloroform–methanol (1:2, v/v) was carried out by the method of Farquhar, Insull, Rosen, Stoffel & Ahrens (1959); 2hr. was sufficient for the complete hydrogenation of phosphatidylcholine, but it was necessary to allow the reaction to continue for at least 24hr. with cardiolipin. The products were purified by running them as strips on thin layers of silica gel H with a solvent of chloroform–methanol–water–acetic acid (135:50:4:1, by vol.) for phosphatidylcholine and chloroform–methanol–water–aq. NH_3 (sp.gr. 0·88) (108:50:5:2·85, by vol.) for cardiolipin, and after location by staining marker bands on either side with I_2 vapour they were eluted with ethanol–chloroform–water (10:3:2, by vol.). The cardiolipin showed considerable decomposition during the reaction, but a band was collected from the t.l.c. plate that ran to the same position as the original cardiolipin (R_F 0·50). Fatty acid analysis of the hydrogenated phospholipids showed the reaction to be complete (Table 1).

Monolayer techniques and apparatus. The methods of determining and recording the surface pressure and interfacial potential of the phospholipid monolayers and the binding of [^{14}C]cytochrome *c* by measurements of surface radioactivity have been described by Quinn & Dawson (1969). The counter sensitivity was improved by using a Nuclear–Chicago type D45 flow counter with a window covered with 6μm.-thick polyethyleneterephthalate (Melinex; Imperial Chemical Industries Ltd., Welwyn Garden City, Herts.). The total number of protein molecules

Table 1. *Fatty acid composition of phospholipid monolayers*

Figures denote the area of the peak as a percentage of the total area of peaks emerging from the column.

	Phosphatidylcholine (egg)		Phosphatidic acid (egg)	Cardiolipin (heart)	
Fatty acid	Original	Hydrogenated		Original	Hydrogenated
$C_{16:0}$	30·9	29·6	29·0	0	3·6
$C_{16:1}$	0	0	0	3·0	0
$C_{18:0}$	12·7	59·2	15·3	0	94·4
$C_{18:1}$	34·2	0	38·4	6·1	0
$C_{18:2}$	12·4	0	13·0	84·4	0
$C_{18:3}$	0	0	0	3·9	0
$C_{20:0}$	0	5·3	0	0	2·1
$C_{20:3}$	0·1	0	0	1·5	0
$C_{20:4}$	4·8	0	4·0	0	0
$C_{22:0}$	0	4·7	0	0	0
$C_{22:6}$	3·4	0	—	0	0

bound to the interface was calculated from the determinations of surface radioactivity as previously described (Quinn & Dawson, 1969). Again in this investigation ΔV refers to the change in surface potential on addition of protein rather than that occurring on spreading the phospholipid film. The changes on addition of protein reported in this investigation refer to the final equilibrium reached in the reaction unless otherwise stated.

RESULTS AND DISCUSSION

Variation of the penetration of cytochrome c *in phospholipid monolayers with protein concentration.* When native cytochrome *c* was added below monolayers of egg phosphatidylcholine at an initial starting pressure of 2dynes/cm. the increase in surface pressure ($\Delta\pi$) at equilibrium plotted against the protein concentration showed evidence of a biphasic relationship (Fig. 1). There was no difference in this pattern whether successive increments of protein were added beneath the same monolayer of phospholipid or increasing amounts of protein were added below individual films at 2dynes/cm. The penetration was initially exponential but eventually became linear when the surface pressure reached 8–9dynes/cm. However, if the starting pressure of the phosphatidylcholine film was 8dynes/cm. the penetration of cytochrome *c* did not show such a linear phase (Fig. 1). Clearly the presence of the penetrated cytochrome *c* in the phosphatidylcholine film (initially at 2dynes/cm.) was influencing the further penetration of additional protein molecules.

The biphasic relationship seen with very expanded films of phosphatidylcholine was not observed in equivalent experiments with phosphatidylethanolamine at the same initial surface pressure of 2dynes/cm. (Quinn & Dawson, 1969). Biphasic or polyphasic relationships in the $\Delta\pi$–protein concentration curve have been observed with the interaction of various proteins and polypeptide hormones with low-pressure films of various non-phosphorus-containing lipids, e.g. stearic acid, cholesterol and dihydroceramide lactoside (Eley & Hedge, 1956, 1957; Colacicco, Rapport & Shapiro, 1967; Snart & Sanyal, 1968). These have been interpreted as representing the build-up of multilayers of protein on the lipid/water interface with the protein molecules showing various degrees of unfolding from the fully denatured to the native protein.

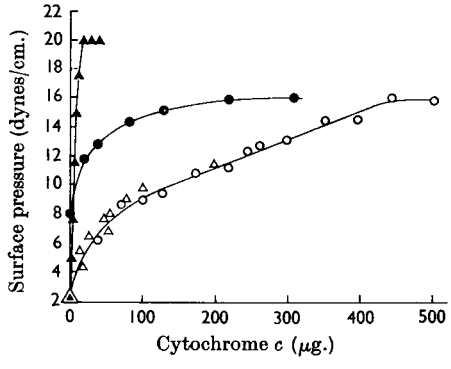

Fig. 1. Penetration ($\Delta\pi$) of cytochrome *c* into phosphatidylcholine and cardiolipin monolayers on a 10mM-NaCl subphase. ●, Phosphatidylcholine (8dynes/cm.); ○, phosphatidylcholine (2dynes/cm.) (successive increments of protein added under the same monolayer); △, phosphatidylcholine (2dynes/cm.) (individual monolayers with single injection of protein); ▲, cardiolipin (2dynes/cm.).

The penetration of cytochrome *c* ($\Delta\pi$) into expanded films of cardiolipin (2dynes/cm.) was markedly greater than that into phosphatidylcholine monolayers, reaching a maximum final pressure of 20dynes/cm. when only 15μg. of cytochrome *c* has been added to the subphase (Fig. 1). The final pressures reached with both phosphatidylcholine and cardiolipin films were above the collapse pressure of the cytochrome *c* itself at the air/water interface (13dynes/cm.). The facilitated penetration into the cardiolipin film seems likely to be due to the electrostatic interactions between the net positively charged protein and the net negative charges on the cardiolipin/water interface, in contrast with the net zero charge on the zwitterionic phosphatidylcholine. Calculation shows that, if all the protein added (15μg.) was associated with the cardiolipin film (see below), each molecule would occupy an area of 1102Å^2 of the suface. This can be compared with an area of 1160Å^2 for each cardiolipin molecule in the film, or 80Å^2 for each negative charge, assuming full ionization of the phospholipid. Thus each protein molecule, which possesses an excess of about 11–14 positive charges in this unbuffered system, is associated with 14 negative charges on the lipid interface. Matalon & Schulman (1949) showed that the penetrations of haemoglobin, γ-globulin and bovine serum albumin were enhanced if the net charge on the protein was opposite to that on a cardiolipin monolayer.

Variation of cytochrome c *interaction with the initial pressure of phospholipid monolayer.* Fig. 2 shows that, as the initial starting pressure of a monolayer of either phosphatidylcholine or cardiolipin was increased, the pressure increment on addition of a constant amount of cytochrome *c* decreased proportionally. Similar patterns have been observed with the interaction of cytochrome *c* and phosphatidylethanolamine monolayers (Quinn & Dawson, 1969), γ-globulin and dihydroceramide lactoside monolayers (Colacicco *et al.* 1967) and the apoprotein of high-density plasma lipoprotein with phosphatidylcholine (Camejo, Colacicco & Rapport,

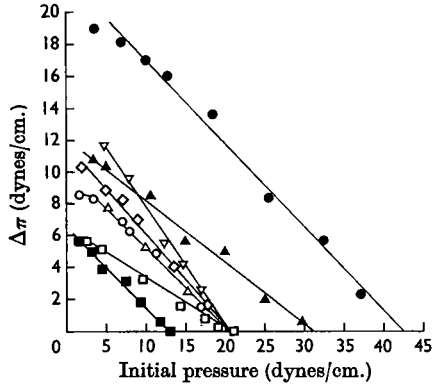

Fig. 2. Surface-pressure increments on addition of cytochrome *c* below monolayers of phosphatidylcholine and cardiolipin at various starting pressures. Cardiolipin monolayers on: ●, 10mM-NaCl; ▲, M-NaCl (50 μg. of cytochrome *c* added to each). Egg phosphatidylcholine monolayers on: ○, 10mM-NaCl; △, M-NaCl (90 μg. of cytochrome *c* added to each); ◇, 10mM-NaCl; ▽, M-NaCl (90 μg. of carboxymethylated cytochrome *c* added to each). Hydrogenated egg phosphatidylcholine monolayer on: □, 10mM-NaCl (90 μg. of cytochrome *c* added). (Dipalmitoyl) phosphatidylcholine on: ■, 10mM-NaCl (90 μg. of cytochrome *c* added).

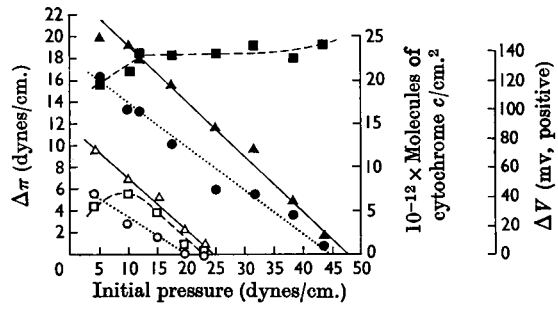

Fig. 3. Binding of [^{14}C]cytochrome *c* to monolayers of phosphatidic acid at various initial starting pressures (33 μg. of protein added). ▲, ■ and ●, Subphase 10mM-NaCl; △, □ and ○, subphase M-NaCl. ▲ and △, Change in surface pressure ($\Delta\pi$); ■ and □, molecules of cytochrome *c*/cm.^{2} of monolayer; ● and ○, change in surface potential on addition of protein (ΔV).

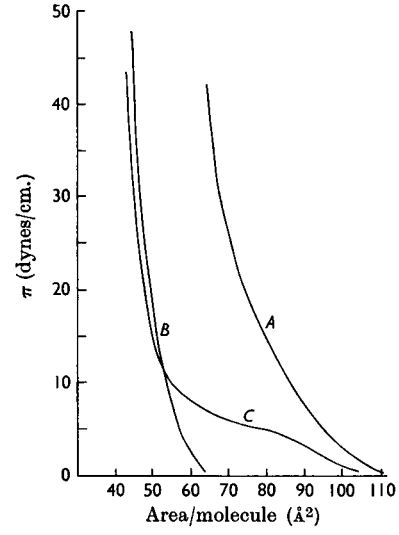

Fig. 4. Area/molecule of phosphatidylcholine at various surface pressures (temperature 17°, subphase 10mM-NaCl). Curve *A*, native egg phosphatidylcholine; curve *B*, hydrogenated phosphatidylcholine; curve *C*, (dipalmitoyl) phosphatidylcholine.

1968). Penetration of cytochrome *c* into phosphatidylcholine monolayers ceased above initial pressures of 20dynes/cm., whereas with cardiolipin films on 10mM-sodium chloride penetration occurred almost up to the collapse pressure of the film. With phosphatidylethanolamine monolayers penetration ceased above 24dynes/cm. (Quinn & Dawson, 1969), again suggesting that the degree of penetration was controlled by the number of negative sites on the film. Thus it is to be expected that on the unbuffered subphase used phosphatidylethanolamine molecules would be partially negatively charged owing to some deprotonization of the amino groups and full ionization of the phosphate moiety, and that the negative charge density on these films would be intermediate between those on monolayers of cardiolipin and zwitterionic phosphatidylcholine.

If the interaction was performed in the presence of high concentrations of sodium chloride (1M) the penetration into phosphatidylcholine monolayers was unaffected, whereas the penetration into cardiolipin did not occur above 30dynes/cm. (Fig. 2) and into phosphatidylethanolamine above 19dynes/cm. This again emphasizes that penetration of the protein into monolayers of an acidic phospholipid was being facilitated by electrostatic forces, which were decreased in the presence of high salt concentrations. A similar effect of salt was seen with the penetration of cytochrome *c* into phosphatidic acid monolayers (Fig. 3), where the addition of M-sodium chloride decreased the threshold of penetration from 47dynes/cm. (collapse) to 24dynes/cm., i.e. just above that observed with a phosphatidylcholine monolayer. The complete hydrogenation of egg phosphatidylcholine caused a decrease in the slope of the plot of penetration against the initial starting pressure of the monolayer, but the minimum initial pressure at which no penetration occurred remained

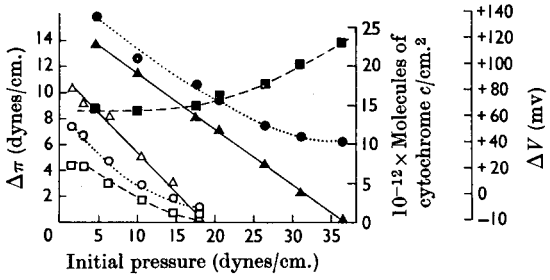

Fig. 5. Adsorption of [14C]cytochrome c on monolayers of phosphatidylcholine and cardiolipin at various initial surface pressures. △, □ and ○, Phosphatidylcholine (90 μg. of protein added); ▲, ■ and ●, cardiolipin (hydrogenated) (33 μg. of protein added). △ and ▲, change in surface pressure (Δπ); □ and ■, molecules of cytochrome c/cm.² of monolayer; ○ and ●, surface potential (ΔV).

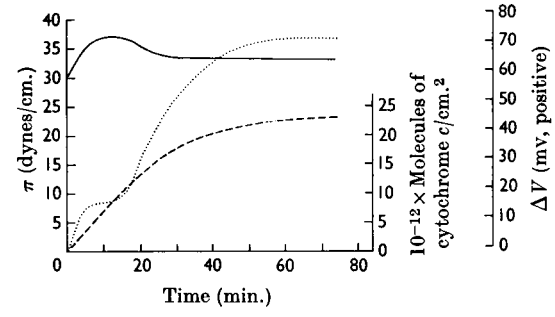

Fig. 6. Time-course, taken from a recorder tracing, of the interaction of a monolayer of native cardiolipin on a subphase of 10mM-NaCl with [14C]cytochrome c. A 33 μg. sample of [14C]cytochrome c was added to the subphase at zero time and the surface pressure (π) (——), change in the surface potential (ΔV) (.....) and the number of cytochrome c molecules in the surface phase (----) were recorded.

the same (Fig. 2). A similar result was obtained with phosphatidylethanolamine (Quinn & Dawson, 1969). Since the density of hydrogenated phosphatidylcholine molecules per unit area of surface would be much greater at this constant threshold pressure than that of the native egg phosphatidylcholine (Fig. 4), this again suggests that the limiting pressure for penetration depends on the work necessary for penetration to occur rather than on the theoretical space available between the phospholipid molecules. When penetration of protein into the film is possible energetically, the decrease in the amount of space available between the molecules caused by hydrogenation decreases the magnitude of the penetration (Fig. 2).

Although the threshold pressure at which penetration of cytochrome c into cardiolipin monolayers ceased appeared to be slightly decreased by hydrogenation of the phospholipid (cf. Figs. 2 and 5), the situation is complicated by the anomalous behaviour of native cardiolipin monolayers at high pressures (> 25 dynes/cm.). Thus on addition of cytochrome c below such films, the surface pressure increased for about 10 min. and then declined somewhat; a similar irregularity was seen in the interfacial potential–time curves (Fig. 6). Although it is possible that this decline might be caused by cardiolipin leaving the monolayer, there was no evidence from measurements of surface radioactivity that there was any similar disturbance in the rate of total protein binding. This could mean therefore that the cardiolipin–cytochrome c complex is undergoing some time-dependent conformation change caused by the protein interacting with the unsaturated fatty acid residues of the phospholipid, thus decreasing their freedom of movement and kinetic energy. Hydrogenated cardiolipin, where the motion of the fatty acid chains is already restricted, did not show this anomalous behaviour during penetration of cytochrome c, and it was not observed with monolayers of phosphatidylcholine or phosphatidic acid. Although hydrogenation of egg phosphatidylcholine did not change the minimum initial surface pressure for penetration of cytochrome c, there was an appreciable lowering in the threshold pressure with monolayers of (dipalmitoyl) phosphatidylcholine (Fig. 2). This is clearly not due to any change in the packing density of the phospholipid molecules, because at 20 dynes/cm. the areas per molecule of hydrogenated egg phosphatidylcholine and (dipalmitoyl) phosphatidylcholine are practically identical (Fig. 4), both being in the fully condensed phase at this temperature (Phillips & Chapman, 1968). It is possible that the difference could be ascribed to the longer average chain length of the hydrogenated egg phosphatidylcholine fatty acids. Differences have been observed in the penetration of serum albumin into monolayers of phosphatidylcholine possessing fatty acids of different chain lengths (P. J. Quinn & R. M. C. Dawson, unpublished work).

The degree of carboxymethylation of cytochrome c involved in its labelling did not affect the threshold pressure at which penetration ceased, but the slope of the Δπ–initial film pressure plot was increased, and the presence of M-sodium chloride increased the slope still further (Fig. 2). This effect is seen in more detail in Fig. 7, which shows the relationship between increments in the surface pressure (Δπ), surface potential (ΔV) and surface radioactivity (ΔR) on addition of a constant amount of cytochrome c, with various proportions of [14C]carboxymethylated and native components, below (dipalmitoyl) phosphatidylcholine monolayers. If the

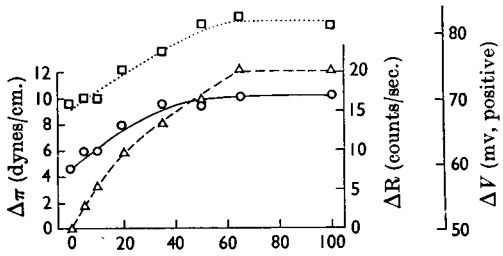

Fig. 7. Comparison of the adsorption of native and [^{14}C]-carboxymethylated cytochrome *c* on (dipalmitoyl) phosphatidylcholine monolayers. The total amount of protein added was 90 μg. and the percentage of the carboxymethylated form was varied. The initial pressure of the monolayer was 5 dynes/cm. Changes in surface potential (ΔV) (□), surface pressure ($\Delta\pi$) (○) and surface radioactivity (ΔR) (△) on addition of the protein were recorded.

reactions of the two types of protein were indistinguishable the $\Delta\pi$ and ΔV plots would have been horizontal and the surface radioactivity a straight line starting at zero (i.e. that for native cytochrome *c*). The alkylated protein clearly has a greater final affinity for the phosphatidylcholine monolayer, and this difference was also reflected in the rate of reaction, which was appreciably faster for the carboxymethylated protein. This was in marked contrast with the penetration into phosphatidylethanolamine monolayers, where no difference was observed (Quinn & Dawson, 1969). Similar experiments with monolayers of cardiolipin and phosphatidic acid have also shown no detectable difference between the penetration of the native and the carboxymethylated cytochrome *c*. This could suggest that differences in penetration are not observed when the interface has a net surplus of anionic sites. Perhaps the electrostatic forces between the interface and the protein, being the most powerful, predominate and mask any differential selection, which is therefore only seen during the reaction with zwitterionic phosphatidylcholine monolayers.

Observations on the amount of [^{14}C]cytochrome *c* in the surface phase indicated that with phosphatidylcholine monolayers the total number of molecules of bound protein was directly related to the penetration (Fig. 5). This indicates that over the pressure range where penetration occurs the number of molecules of cytochrome *c* entering the phospholipid film is proportional to $\Delta\pi$ and that there are not differing degrees of penetration of the same number of molecules. No adsorption of the labelled cytochrome *c* occurred at high initial film pressures in the absence of penetration. As noted previously (Quinn & Dawson, 1969) with phosphatidylethanolamine monolayers, at all pressures the protein penetrating the phosphatidylcholine monolayers represented only a small percentage of that added to the system.

When cytochrome *c* had interacted with a film of phosphatidylcholine at 2 dynes/cm. on either 10 mM- or M-sodium chloride it could all be removed by compression of the film to 32 dynes/cm. (i.e. above the initial surface pressure when no penetration would occur). This is in contrast with the interaction of cytochrome *c* with phosphatidylethanolamine monolayers, where the penetrated protein was only desorbed on compression when the film was on a subphase of M-sodium chloride (Quinn & Dawson, 1969). This therefore suggests that non-electrostatically facilitated penetration is largely reversible on compression of the film whereas electrostatically stabilized penetration is irreversible.

Adsorption of cytochrome c *on acidic phospholipid monolayers independent of penetration.* With cardiolipin and phosphatidic acid monolayers the total binding of [^{14}C]cytochrome *c* was, unlike that of phosphatidylcholine, not proportional to penetration but increased as the initial starting pressure of the film was increased (Figs. 3 and 5). Clearly adsorption of cytochrome *c* on the film was occurring that could not be accounted for by the penetration, and the surface radioactivity represents the sum of that arising from penetrated and 'adsorbed' protein. The total amount of protein bound to the surface appeared to increase as the charge density of the negatively charged phospholipid molecules on the surface was increased by compression. The added protein (33 μg.) produced a maximal surface radioactivity with phosphatidic acid monolayers at about 12 dynes/cm. (Fig. 3), and calculation showed that at this pressure all the added protein was present on the surface. On the other hand, with cardiolipin monolayers spread on the same area of the trough, complete binding of the protein was not observed until about 37 dynes/cm. (Fig. 5). However, comparison of the π–area/molecule curves of the two phospholipids at the pressures where maximal radioactivities were achieved indicated a close similarity in the area that each molecule occupied on the surface (84 Å^2 for phosphatidic acid, 89 Å^2 for cardiolipin). If complete dissociation of all ionogenic groups is assumed, the surface charge densities would therefore be equal. Although it might be expected that full ionization of phosphatidic acid would only occur at higher pH values there is evidence that in the presence of adsorbed cations, e.g. Ca^{2+}, the ionization occurs at lower pH values of the bulk phase (Hauser & Dawson, 1967). It seems possible that the adsorption of the basic cytochrome *c* might also initiate the ionization of the second phosphate group at a lower pH value, and this is indeed indicated by a comparison of the

changes in surface potentials on the interaction of cytochrome c with phosphatidic acid monolayers at various pH values and the surface potentials of the monolayers before interaction with the protein.

The surface potential changes on addition of protein generally reflected the penetration of the protein into the film (Figs. 3 and 5), and with phosphatidic acid monolayers the adsorbed non-penetrated protein did not result in a change of the interfacial potential (Fig. 3). When the protein was adsorbed on monolayers of hydrogenated cardiolipin at high pressure there was an increase (positive) in the surface potential, but this was not observed when cytochrome c interacted with the native phospholipid.

When the binding of [14C]cytochrome c to a mixed monolayer of phosphatidylcholine and cardiolipin (92·5/7·5 molar proportions) was examined at various initial pressures the affinity was intermediate between that of films of pure cardiolipin and phosphatidylcholine (Fig. 8). Thus [14C]-cytochrome c penetrated the film up to a maximum pressure of 29dynes/cm. compared with phosphatidylcholine at 20dynes/cm. and cardiolipin at 42dynes/cm. Similarly, the addition of cardiolipin to the phosphatidylcholine allowed some adsorption on high-pressure films in the absence of penetration, but this was considerably less than with cardiolipin alone.

Displacement of cytochrome c *from phospholipid monolayers by addition of* M*-sodium chloride.* Cytochrome c that had interacted with a phosphatidylcholine monolayer at low film pressures (< 20dynes/cm.) was not removed by increasing the subphase sodium chloride concentration from 10mM to 1M. This is to be expected from the results given above, which suggested that the penetration into phosphatidylcholine films was not facilitated by electrostatic forces, and that no electrostatically motivated adsorption independent of penetration

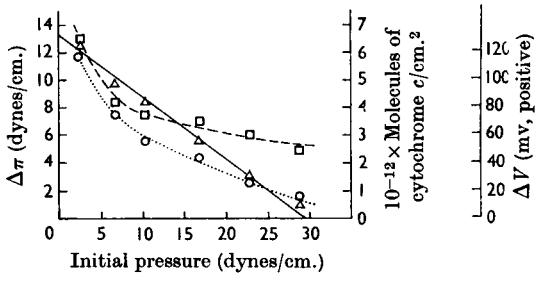

Fig. 8. Interaction of [14C]cytochrome c and mixed monolayers of phosphatidylcholine and cardiolipin (92·5/7·5 molar ratio). The increment in surface pressure ($\Delta\pi$) (△), surface potential (○) and surface radioactivity (expressed as molecules of protein/cm.²) (□) on addition of [14C]cytochrome c (33μg.) were recorded.

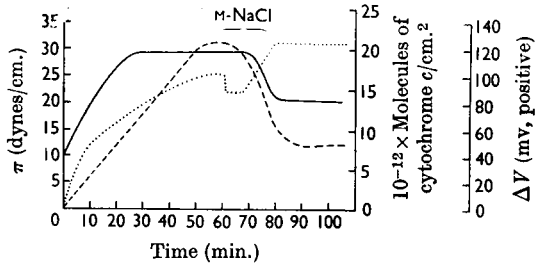

Fig. 9. Time-course, taken from a recorder tracing, of the interaction of [14C]cytochrome c with a monolayer of phosphatidic acid spread on a subphase of 10mM-NaCl and subsequent changes in surface pressure (π) (——), surface potential (ΔV) (.....) and the concentration of the protein in the surface phase (– – – –) on addition of NaCl to the subphase, to a final concentration of 1M.

occurred. Fig. 9 shows the effect of adding sodium chloride to the subphase after [14C]cytochrome c had interacted with a phosphatidic acid film at 10dynes/cm. At this pressure M-sodium chloride produced no appreciable contraction of a phosphatidic acid monolayer alone, although the interfacial potential was changed owing to alteration of the ionic dipoles by the counterion binding of Na^+. With interacted lipoprotein films, M-sodium chloride displaced a proportion of the penetrated protein, i.e. the pressure was decreased by 9dynes/cm. compared with the 12dynes/cm. that would be expected from the values presented in Fig. 3, which gives the equilibrium positions of the interactions on subphases of 10mM- and M-sodium chloride. The displacement of surface radioactivity suggested that, as well as the removal of part of the penetrated protein, a substantial proportion of the bound non-penetrated protein had also been displaced.

The addition of M-sodium chloride to a system in which cytochrome c had been allowed to interact for 60min. with a high-pressure phosphatidic acid monolayer (45dynes/cm.) in which penetration would be minimal (Fig. 3) again showed a substantial (65%) displacement of the non-penetrated protein. This contrasts with the interaction of [14C]cytochrome c with phosphatidylethanolamine monolayers, where, although initially most of the non-penetrated cytochrome c was removed by addition of M-sodium chloride after 40min. only 20% was removed (Quinn & Dawson, 1969). It was suggested that this reaction might represent a non-ionic interaction of the protein with the ethanolamine moiety, and its absence with phosphatidic acid supports this contention. The addition of sodium chloride to the system caused a slight decrease in the surface pressure, but the interpretation of this is complicated by the observation that M-sodium chloride caused a contraction of high-pressure phosphatidic

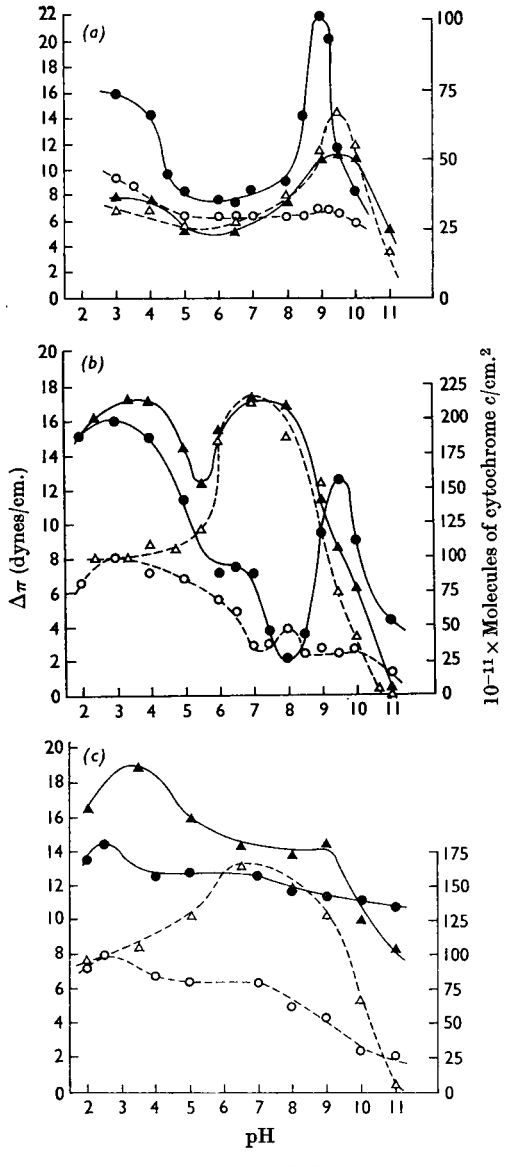

Fig. 10. Effect of pH of the subphase on the interaction between [^{14}C]cytochrome *c* and monolayers of (*a*) egg phosphatidylcholine, (*b*) egg phosphatidic acid and (*c*) native cardiolipin at initial pressures of 10 dynes/cm. The protein (90 μg. for *a*, 33 μg. for *b* and *c*) was introduced into the subphase (75 ml.), the pH being maintained with NaOH or HCl regulated by a pH-stat. ▲ and △, Subphase 10 mM-NaCl; ● and ○, subphase M-NaCl. ▲ and ●, Changes in surface pressure ($\Delta\pi$); △ and ○, changes in the number of cytochrome *c* molecules in the surface phase.

acid monolayers in the absence of protein, presumably by minimizing the electrostatic repulsion between adjacent polar head groups.

Effect of pH *of the subphase on the interaction of* [^{14}C]*cytochrome* c *with phospholipid monolayers.* The following discussion is generally concerned with the final equilibrium reaction of cytochrome *c* with phospholipid films at various subphase pH values, although occasional reference will be made to the initial rate of the interaction. The total number of cytochrome *c* molecules reacting with phosphatidylcholine monolayers at initial pressures of 10 dynes/cm. remained relatively constant between pH 3 and 8 (Fig. 10*a*) and the difference between the effects on subphases of 10 mM-sodium chloride and M-sodium chloride is probably due to carboxymethylation of the cytochrome *c* (Fig. 2). This is consistent with the phosphatidylcholine head group acting as a zwitterion between pH 3 and 10, deduced from surface potential–pH plots (Fig. 11*a*) and particle-microelectrophoresis studies (Bangham & Dawson, 1959). However, the increment in surface pressure, $\Delta\pi$, on penetration was higher below pH 4, especially in the presence of M-sodium chloride. The decline in $\Delta\pi$ above pH 4 appears to correspond to the ionization of carboxyl groups of the protein (Fig. 11*b*), but if the correlation is real it is difficult to understand why the magnitude is larger in the presence of salt, which might be expected to decrease the effect of the ionization. At low salt concentrations more cytochrome *c* reacted with phosphatidylcholine films at pH values just below the isoelectric point (10·25) of the protein, reaching a maximum at pH 9·5 (Fig. 10*a*), although the initial rate of the reaction remained relatively constant. This increase was reflected in a similar change in the radioactivity increments and correlates well with an increase in the limiting area of a monolayer of cytochrome *c* spread at an air/10 mM-sodium chloride interface (Fig. 11*c*). Clearly under these conditions there is a conformation change in the protein at about pH 9·5 that results in a greater affinity for a phosphatidylcholine monolayer. There is, however, no indication that the extra protein molecules associated with the monolayer at pH 9·5 are interacting with protein molecules already penetrating the film. Thus, on spreading monolayers of native cytochrome *c* at the air/water interface at this pH, no binding of [^{14}C]cytochrome *c* introduced into the subphase was observed. This is consistent with earlier observations on the weakness of intermolecular forces between adsorbed and bulk-phase cytochrome *c* molecules, based on particle-microelectrophoresis studies (Quinn & Dawson, 1969).

The reaction with phosphatidylcholine films on M-sodium chloride was attended by a pronounced increase in $\Delta\pi$ at about pH 9 (Fig. 10*a*) that was not detected in 10 mM-sodium chloride. The measurements of surface radioactivity showed that this was not accompanied by an increase in the number of protein molecules bound to the interface. Clearly a

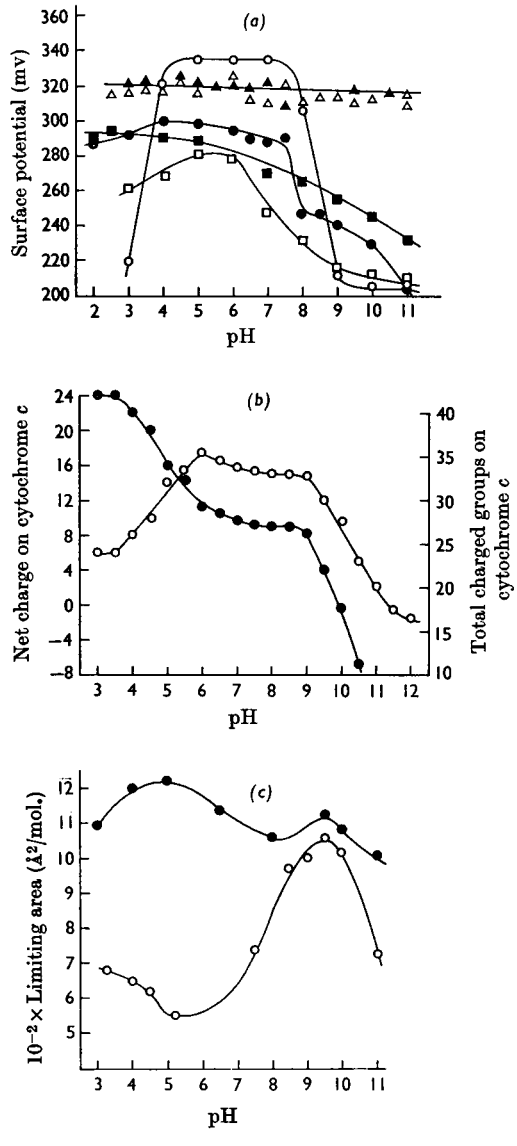

Fig. 11. (*a*) Relationship between pH of the subphase and surface potentials of phospholipid monolayers on subphases of 10mM-NaCl (△, ○ and □) and M-NaCl (▲, ● and ■) and adjusted to a surface pressure of 10dynes/cm. △ and ▲, Egg phosphatidylcholine; ○ and ●, egg phosphatidic acid; □ and ■, native cardiolipin. The pH of the subphase was adjusted with NaOH or HCl and maintained with a pH-stat. (*b*) Theoretical calculation of the net charge (●) and total number of charged groups (○) of cytochrome *c* at pH values between 3 and 12 based on the p*K* values for protein ionogenic groups given by Tanford & Hauenstein (1956) and the amino acid sequence given by Margoliash & Schejter (1966). The curves agree well with the titration curves of Paul (1951). (*c*) Limiting area/mol. of native cytochrome *c* spread on subphases of 10mM-NaCl (○) and M-NaCl (●) from a solution of the protein in aq. 30% (v/v) ethanol. The limiting area was obtained by extrapolation from the linear region of the surface pressure–area/molecule curve obtained by compression of an expanded monolayer of the protein. The subphase was stirred during these measurements since otherwise it was found that with ^{14}C-labelled protein films with an area/molecule greater than the limiting area there was a slow loss of radioactivity. This was interpreted as protein moving to the edges of the trough and away from the counter, since on compression or restirring of the film all radioactivity was recovered.

greater space-occupying part of each protein molecule is penetrating the film at this pH, resulting in a final film pressure of about 32dynes/cm. A similar but slightly less pronounced increase in the penetration of cytochrome *c* into phosphatidylethanolamine monolayers on M-sodium chloride was observed at pH9 (Quinn & Dawson, 1969). The increased penetration is not associated with any marked ionization of groups on either the protein or the phosphatidylcholine (Figs. 11*a* and 11*b*) or with a change in the area occupied by cytochrome *c* at the air/M-sodium chloride interface (Fig. 11*c*). However, a change in the optical-rotatory-dispersion spectrum of cytochrome *c* at pH values between 7 and 9·5 has been interpreted as a change in the conformation around the haem region (Mirsky & George, 1966, 1967), and this might be connected with the enhanced penetration of the cytochrome *c* molecule into the monolayer.

The penetration of cytochrome *c* into phosphatidic acid monolayers at 10dynes/cm. follows a complicated relationship to the pH of the bulk phase (Fig. 10*b*). The $\Delta\pi$ in the presence of 10mM-sodium chloride at all pH values below 9 was always greater than with M-sodium chloride. This can be contrasted with the reverse situation existing with phosphatidylcholine monolayers, and is probably a consequence of the electrostatically facilitated penetration into phosphatidic acid films that is prevented by high salt concentrations. The $\Delta\pi$ peak at pH3·5 is coincident with ionization of the first phosphate group of the phospholipid, as shown in ΔV–pH plots of phosphatidic acid monolayers (Fig. 11*a*). Between pH3·5 and 5·5 the penetration into the phosphatidic acid monolayer fell and, as with phosphatidylcholine, this might be related to the ionization of carboxyl groups on the protein (Fig. 11*b*). Above pH5·5 M-sodium chloride produced a very marked difference in both the total number of protein molecules in the surface phase and their penetration into the monolayer. The increase in both $\Delta\pi$ and surface radioactivity in low salt concentrations preceded the ionization of the second phosphate group, judged by the ΔV–pH plots of phosphatidic acid monolayers (Fig. 11*a*). However, ΔV–pH plots in the presence of protein

suggest that this ionization may occur at a somewhat lower pH (pK 7–8). This ionization appears to be associated with a marked trough in the penetration of cytochrome *c* into phosphatidic acid films on M-sodium chloride (Fig. 10*b*). The greater surface-radioactivity increment on 10 mM-sodium chloride compared with that on M-sodium chloride at pH values between 4 and 10 was not due to any contraction of the film brought about by the salt, as similar differences were observed in experiments at constant area/molecule. It is probably partly a consequence of electrostatically controlled adsorption of non-penetrated protein on the monolayers of phosphatidic acid, as between these pH values the net charge on the protein will be opposite to that on the monolayer. The electrostatically facilitated penetration mentioned above (Fig. 3) may also contribute to the increase in the number of cytochrome *c* molecules bound to the phosphatidic acid monolayers on 10 mM-sodium chloride, although it cannot be ascertained from the results whether this is due to a greater percentage of the protein molecules penetrating into the monolayer or to a higher proportion of each molecule entering the film.

A similar relationship between the total amount of interacted protein and pH was found with cardiolipin monolayers, where the total amount of protein bound was decreased between pH 2·5 and 10 by addition of M-sodium chloride (Fig. 10*c*). This again is probably due to both electrostatically facilitated penetration and electrostatic adsorption in the absence of penetration. Again, below the isoelectric point of the protein (10·05) penetration was hindered by M-sodium chloride, whereas above this pH, when the protein acquires the same net charge as the film, the penetration was assisted by M-sodium chloride. The $\Delta\pi$–pH curve in M-sodium chloride approximately followed the curve of the total number of protein molecules bound to the cardiolipin monolayer, although there is some evidence that at higher pH values a greater proportion of each protein molecule was entering the film (Fig. 10*c*).

With all phospholipid films the discharge of the lysine residues on the cytochrome *c* molecule (Fig. 11*b*) coincided with a dramatic decrease in the number of protein molecules at the interface. With the acidic phospholipids this process began before the net charges on the phospholipid and cytochrome *c* molecules had become of similar sign.

The variation of the interaction between cytochrome *c* and a phospholipid surface with the pH of the subphase can clearly only be partially understood from the gross electrostatic factors that prevail, i.e. the net charges of the protein and phospholipid. Although consideration of the dissociation of each type of ionogenic group on both the phospholipid and the protein helps this understanding, other subtle changes, presumably in the conformation of the protein, considerably influence the magnitude and stability of the association. It is apparent that the often-made assumption that the complex-formation between cytochrome *c* and phospholipids can be described in purely electrostatic terms is an oversimplification.

Binding of cytochrome c *in mitochondria.* Green & Fleischer (1963) suggested that the cytochrome *c* in mitochondria was bonded electrostatically to anionic phospholipids. The ready displacement of the protein from mitochondria by saline solutions (Jacobs & Sanadi, 1960; González-Cadavid & Campbell, 1967) after they had been subjected to hypo-osmotic conditions would suggest that some form of electrostatic bonding is present, although it has to be remembered that this could also involve a protein–protein interaction with mitochondrial structural protein (Edwards & Criddle, 1966). Cytochrome *c*-depleted mitochondria can take up exogenous cytochrome *c* by a process that amounts to titration of the vacant binding sites, the protein being completely removed from solution up to the point of saturation (Jacobs & Sanadi, 1960). The present investigations suggest that if cytochrome *c* is bound to a phospholipid in the mitchondria the most likely candidate would be cardiolipin. This is indicated by the capacity of monolayers of cardiolipin to take up quantitatively cytochrome *c* added to the subphase, compared with phosphatidylethanolamine and phosphatidylcholine monolayers, which bind only a small percentage of the cytochrome *c* added to the subphase. The cytochrome *c* associated with cardiolipin films is largely displaced by high concentrations of sodium chloride, leaving only a small residue that is presumably non-ionically bound. Even in mitochondria there is a small component of the endogenous cytochrome *c* that is not removed by saline solutions (MacLennan, Lenaz & Szarkowska, 1966). It is perhaps not a coincidence that the cytochrome *c* content of mammalian tissues appears to be related to the cardiolipin content (Getz, Bartley, Lurie & Notton, 1968).

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