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Interaction of Cytochrome *c* with Phospholipid Monolayers and Bilayer Membranes

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Summary. For the study of the interaction between oxidized cytochrome *c* and phosphatidylinositide, two different model systems were used: (1) monolayers which were deposited after the method of Langmuir and Blodgett onto glass plates, and (2) bimolecular ("black") membranes in aqueous phase. The amount of bound protein was determined with a sensitive spectrophotometer. It was found that at low ionic strength about 10^{13} cytochrome *c* molecules per cm^2 are bound to the lipid surface, which nearly corresponds to a densely packed monolayer. At high ionic strength ($\sim$0.1 M) or low pH (pH < 3), the adsorbed protein layer becomes unstable. This result indicates that the interaction is mainly electrostatic. In accordance with this conclusion is the observation that the rate of adsorption is diffusion controlled; i.e., almost every protein molecule hitting the surface is bound. The cytochrome *c* monolayer can be reduced by ascorbate. In contrast to ferrocytochrome *c* in solution, the bound ferrocytochrome was found to be autoxidable.

Among the mitochondrial proteins, cytochrome *c* occupies an intermediate position between the "soluble" proteins which are localized in the matrix space and the proteins from which the membrane is built up. Upon ultrasonic fragmentation of the mitochondrion, the cytochrome *c* remains bound to the inner membrane; on the other hand, it may be extracted from osmotically shocked mitochondria by aqueous salt solutions. These, as well as other observations, may be explained by the assumption that cytochrome *c* is bound to the outside of the inner mitochondrial membrane mainly by electrostatic interactions (Nicholls, Mochan & Kimelberg, 1969). This hypothesis is supported by the results of several experimental studies with model systems. Cytochrome *c* (which bears a net positive charge at pH < 10.6), forms a hydrocarbon-soluble complex with acidic phospholipids (Das, Haak & Crane, 1965; Ulmer, Vallee, Gorchein & Neuberger, 1965; Sun & Crane, 1969; Hart, Leslie, Davies & Lawrence, 1969; Shipley, Leslie & Chapman, 1969*a*). Besides this, it has been shown by

X-ray diffraction techniques (Papahadjopoulos & Miller, 1967; Shipley, Leslie & Chapman, 1969*b*; Gulik-Krzywicki, Shechter, Luzzati & Faure, 1969) and by electron microscopy (Kimelberg, Lee, Claude & Mrena, 1970) that cytochrome *c* may be incorporated into the polar layers of liquid crystals prepared from negatively charged phospholipids. Kimelberg *et al.* (1970) observed that the interaction becomes very weak at ionic strengths above 0.1 M. A similar result has been obtained by Quinn and Dawson (1969*a*, *b*) from studies with phospholipid monolayers at the water-air interphase. However, these authors were able to demonstrate that under certain conditions (low film pressure) the cytochrome may also be bound via nonelectrostatic interactions.

In this paper we present a study of two further models which offer certain advantages compared with the previously described systems. As a convenient method for the preparation of a well-defined lipid surface, the Langmuir-Blodgett technique has been used by which a lipid monolayer can be transferred from the water surface onto a solid substrate such as, for instance, a glass plate. The binding of cytochrome *c* to the lipid surface has been followed with a sensitive spectrophotometer. In a second series of experiments, the interaction of cytochrome *c* with lipid bilayer membranes in aqueous phase was studied. In this case, the spectrophotometric determination of the bound protein may be combined with electrochemical studies.

Materials and Methods

Materials

Oxidized cytochrome *c* (horse heart, Type III) was obtained from Sigma. Because it is known that preparations of cytochrome *c* occasionally contain polymeric forms of the protein (*see* Margoliash & Lustgarten, 1962; Havez, Hayem-Lévy, Mizon & Biserte, 1966), the commercial product was analyzed on a Sephadex G 75 column (Margoliash & Lustgarten, 1962). Because we found that the amount of polymeric material was less than 1%, the product was used without further purification.

Phosphatidylinositide was prepared from yeast by the method of Trevelyan (1966). Later (for the bilayer experiments) a commercial product (Koch-light) was used. Dioleoyllecithin was obtained by acylation of glycerylphosphorylcholine with oleic anhydride (Robles & van den Berg, 1969). The purity of the lipids was checked by thin layer chromatography.

Deposition of Lipid Monolayers

For the deposition of lipid monolayers onto glass plates, the technique of Langmuir and Blodgett was used (*see* Gaines, 1966; Bücher, v. Elsner, Möbius, Tillmann & Wiegand, 1969). The trough for the preparation of monolayers at the air-water interphase was made of lucite and was equipped with a motor-driven movable barrier. The lipids were spread from benzene solution on an aqueous solution of 0.03 mM $BaCl_2$ + 0.4 mM

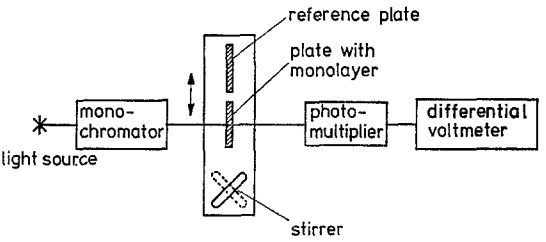

Fig. 1. Single-beam spectrophotometer for the study of thin pigment films

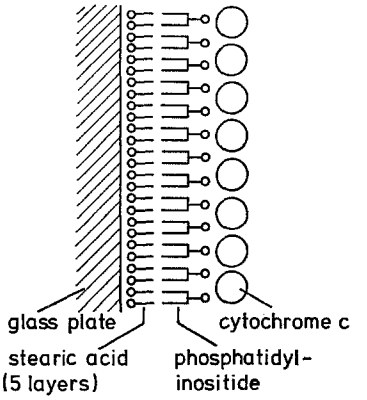

Fig. 2. Binding of cytochrome *c* on a phosphatidylinositide monolayer

$KHCO_3$, pH 7.2±0.1. During deposition, the film pressure (measured with a Wilhelmy balance) was held constant at 17.0±0.5 dynes/cm (stearic acid) or 10.0±0.5 dynes/cm (phosphatidylinositide) by adjusting the position of the barrier. An odd number (usually five) of stearic acid layers were first deposited on each side of the glass plate (microscope slides 23 × 55 × 1.25 mm). For this purpose, the plate was submersed in the aqueous phase prior to the formation of the monolayer. When the plate was then pulled upward, a first layer was deposited. A second layer was transferred when the plate was again immersed into the aqueous phase, etc. After the deposition of an odd number of layers, the surface of the plate is hydrophobic, as the hydrocarbon chains of the last layer are directed outward. Onto this hydrophobic surface, a single layer of phosphatidylinositide was deposited with the polar groups outside (Fig. 2). For the binding studies with cytochrome *c*, only freshly coated plates were used.

Bilayer Membranes

Bilayer membranes were produced by the usual method from a 0.5% (w/v) of phosphatidylinositide or dioleoyllecithin in *n*-decane (Läuger, Lesslauer, Marti & Richter, 1967). The membrane was formed on a Teflon frame with a circular hole of 6-mm diameter, which was mounted inside the optical cell of the spectrophotometer.

Spectrophotometric Determination of Bound Cytochrome c

For the determination of the bound cytochrome *c*, a sensitive spectrophotometer was constructed (*see* Fig. 1). The light source consisted of a 50-W halogen-tungsten lamp combined with a Jarrel-Ash monochromator (dispersion 33 A/mm, slit width 0.1 mm). For maximal stability of the light source, the lamp house was water-cooled and the voltage supply of the lamp stabilized to better than 10^{-4} (Hewlett-Packard Power Supply Mod. 6966 A). The light beam passed through the optical cell which was filled with an aqueous buffer solution and which contained the glass plate coated with lipid and cytochrome *c*, as well as a second, uncoated glass plate serving as a reference. Both plates were mounted on a common slide and could be brought alternately into the light beam. The

light intensity was measured with an EMI Mod. 6256 S photomultiplier combined with a John Fluke Mod. 405 B high-voltage power supply (stabilization better than $5 \cdot 10^{-5}$). In order to minimize the shot noise from the photocathode, the full light intensity of the lamp was used and, accordingly, the number of dynodes in the photomultiplier reduced to six. The photomultiplier housing was thermostated. The output of the photomultiplier was measured with a differential voltmeter (John Fluke Mod. 873 A) when first the coated plate and thereafter the reference was in the light beam. Since the photomultiplier signal is proportional to the light intensity, the two voltmeter readings gave directly the extinction $E \equiv \log (J_0/J) \approx 0.434 \ (J_0 - J)/J_0$ (J, J_0 = light intensity after passing the coated plate and the reference, respectively). The limiting value of E, which could be detected with the spectrophotometer was about $5 \cdot 10^{-4}$.

At the beginning of the experiment, the optical cell was filled with pure buffer solution; the extinction of the plate with the lipid monolayer was then measured relative to the reference plate in order to correct for small differences in the reflectance. Subsequently, the cell was filled with a buffered cytochrome *c* solution. After some time, the cytochrome *c* solution was replaced repeatedly with pure buffer solution under constant stirring. The extinction of the cytochrome *c* layer remained constant for at least 1 hr in the pure buffer solution.

For the optical study of bilayer membranes in the presence of cytochrome *c*, an improved version of the spectrophotometer was used which will be described in detail in a subsequent communication. The beam was chopped with a frequency of 70 cps and passed alternately across the membrane and the free solution. By phase-sensitive amplification of the photomultiplier signal, the sensitivity of the photometer could be increased by a factor of about 10 compared with the single-beam version. The thinning process of the membrane could be followed by an additional light source and a low-power microscope. Prior to the formation of the membrane, the measuring beam and the reference beam were optically balanced. In order to avoid errors caused by the slow zero point drift of the instrument, the extinction of the black film was determined from the difference of the amplifier signals before and after the destruction of the membrane at the end of the experiment.

Results

Phosphatidylinositide Monolayers

The time course of the binding of cytochrome *c* at three different concentrations is shown in Fig. 3. At a protein concentration of $c_p = 10^{-5}$ M in the solution, the adsorption is complete in less than 2 min. At the concentrations 10^{-6} and $3 \cdot 10^{-7}$ M, the times for half-saturation are about 3 and 8 min, respectively. Practically the same limiting value of the extinction is reached in each of the three experiments.[1] The mean limiting extinction from seven experiments with $c_p = 10^{-5}$ M is $E = (2.0 \pm 0.1) \cdot 10^{-3}$ at the peak of the Soret band ($\lambda = 410$ nm). With a molar extinction coefficient of $\varepsilon = 106{,}100\ \text{M}^{-1}\ \text{cm}^{-1}$ at 410 nm (Margoliash & Frohwirt, 1959), the

1 In contrast to the results obtained with phosphatidylinositide monolayers, the amount of cytochrome *c* adsorbed on plates covered with stearic acid alone was small and irreproducible.

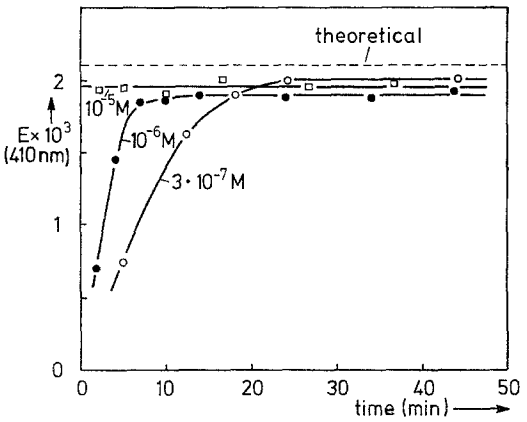

Fig. 3. Time course of the binding of cytochrome *c* on a phosphatidylinositide monolayer for three different concentrations of the protein. *E* is the extinction at the peak of the Soret band ($\lambda = 410$ nm) for *one* layer (measured extinction divided by two). At time $t = 0$, the lipid-coated plate was immersed in a cytochrome *c* solution in 10^{-3} M phosphate buffer, pH = 7.6. From time to time, the adsorption process was shortly interrupted by replacing the cytochrome *c* solution with pure buffer solution. The time on the abscissa is the total time during which the plate was in the protein solution. The dashed line gives the extinction of a densely packed monolayer of cytochrome *c* ($1.2 \cdot 10^{13}$ molecules/cm^2) with random orientation of the porphyrin ring

number of bound cytochrome *c* molecules is calculated to be $N = 1.1 \cdot 10^{13}$ cm^{-2}. The cytochrome *c* molecule is roughly a sphere of diameter $2r = 31$ A (Dickerson, Kopka, Borders, Varnum & Weinzierl, 1967); in a densely packed hexagonal array, the number of cytochrome *c* molecules would, therefore, be $N = 1/2\sqrt{3}\, r^2 \simeq 1.20 \cdot 10^{13}$ cm^{-2} which is only about 10% higher than the experimental value of *N*.

An uncertainty in the calculation of *N* from *E* occurs, however, because it is not clear if the extinction coefficient, ε, of cytochrome *c* in the bound state is the same as in aqueous solution. Two effects have to be considered: (1) ε may change because the molecular environment of the porphyrin ring is different in the two states; (2) the cytochrome *c* layer may be optically anisotropic as a consequence of a preferential orientation of the protein. Point (1) may be excluded because the spectrum of the bound cytochrome *c* very nearly coincides with the solution spectrum (*see* below). However, a preferential orientation of the protein with respect to the lipid monolayer may well occur. This problem will be studied in the near future by polarization experiments. If the transition moment is assumed to be isotropic in the plane of the porphyrin ring (Gouterman, 1961; Eaton & Hochstrasser, 1967), it can be shown that the extreme values of the extinction coefficient

Table. *Number of bound cytochrome c molecules per cm²*

Densely packed hexagonal array	$1.20 \cdot 10^{13}$
Calculated from the measured extinction:	
statistical orientation	$1.13 \cdot 10^{13}$
porphyrin ring parallel to monolayer	$0.75 \cdot 10^{13}$
porphyrin ring perpendicular to monolayer	$1.51 \cdot 10^{13}$

of the cytochrome layer are $\varepsilon_\perp = \frac{3}{4}\varepsilon$ and $\varepsilon_\parallel = \frac{3}{2}\varepsilon$. $\varepsilon_\perp$ and $\varepsilon_\parallel$ are the extinction coefficients for perpendicular and parallel orientation of porphyrin ring relative to the plane of the monolayer, and ε is the extinction coefficient for statistical orientation. The actual number of bound molecules may, therefore, be smaller by a factor of 2/3 or larger by a factor of 4/3 than the number calculated under the assumption of statistical orientation. These different possibilities are summarized in the Table. Despite the uncertainty in the exact value of ε, we may conclude that cytochrome *c* forms nearly a monolayer on the lipid surface over a considerable range of concentrations.

If the interaction of cytochrome *c* with the phosphatidylinositide monolayer is mainly electrostatic, then the binding should become unstable at high ionic strength. Indeed, it was found that the binding became negligible at phosphate buffer concentrations above 0.1 M. This is shown in Fig. 4 in which the extinction is plotted as a function of the phosphate buffer concentration. In a first series of experiments, the adsorption was carried out in a cytochrome solution of a given buffer concentration *c*; after the steady state was reached, the extinction was measured in a pure buffer solution of the same concentration. In a second series of experiments, the protein was bound from a solution of low ionic strength ($c = 10^{-4}$ M) so that the adsorption was maximal. When the plate was then transferred to a buffer solution of $c > 10^{-3}$ M, part of the bound protein was dissolved. Fig. 4 shows that, for a given ionic strength, the extinction is nearly the same in both types of experiment. This means that the interaction is reversible. The same results are obtained if the ionic strength is adjusted by addition of KCl instead of varying the phosphate buffer concentration, indicating that the described effects are true ionic-strength effects and are independent of the specific nature of the electrolyte.

The reversibility of the binding is also evident from another experiment (Fig. 5). Cytochrome *c* is first adsorbed from a buffer solution of concentration 10^{-3} M. After the buffer concentration is changed to 0.15 M, most of the protein is dissolved. (With a buffer solution of ionic strength 1 M,

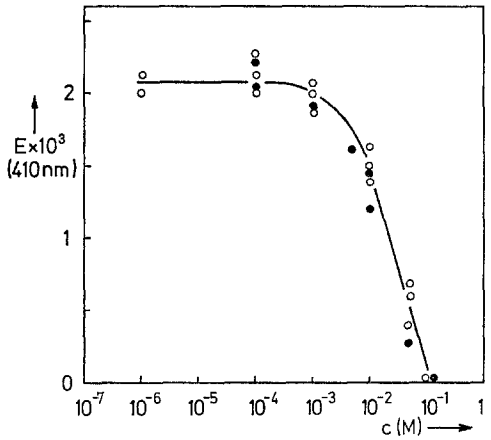

Fig. 4

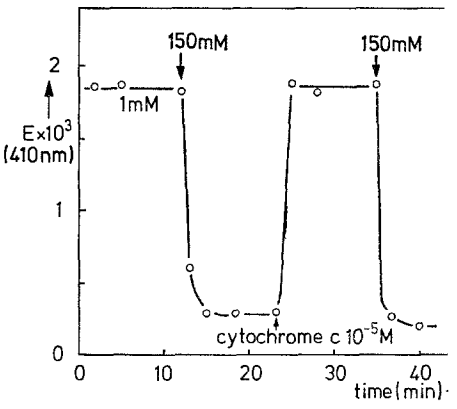

Fig. 5

Fig. 4. Binding of cytochrome *c* on a phosphatidylinositide monolayer as a function of the phosphate buffer concentration *c* (pH = 7.5). *E* extinction of one layer at $\lambda = 410$ nm. ∘, the adsorption was carried out in a cytochrome solution ($c_p = 10^{-5}$ M) in phosphate buffer of concentration *c*, and the extinction measured in pure buffer solution of the same concentration; •, the protein was adsorbed from a 10^{-4} M buffer solution and subsequently transferred to pure buffer solution of concentration *c*

Fig. 5. Reversibility of the binding. At the beginning, the plate with the cytochrome *c* layer is in contact with a phosphate buffer solution of concentration 1 mM (pH = 7.5). After the buffer concentration is changed to 150 mM, most of the protein is dissolved. When the plate is again brought into contact with a cytochrome *c* solution ($c_p = 10^{-5}$ M) in dilute buffer ($c = 1$ mM), the original state is restored

the desorption would be complete.) If the plate is then brought again in contact with a cytochrome *c* solution at low ionic strength, the original extinction is exactly restored. This experiment shows that the phosphatidylinositide layer remains intact during the desorption process. Furthermore, the spectrum of the cytochrome *c* which was extracted from the plate was found to be the same as the normal solution spectrum of the protein.

The conclusion that the interaction is essentially caused by electrostatic forces is further supported by the pH dependence of the binding. Cytochrome *c* bears a net positive charge at pH values below 10.7, whereas phosphatidylinositide is negatively charged for pH ≧ 3.5 (Papahadjopoulos, 1968). It was found that the binding of cytochrome *c* on the phosphatidylinositide monolayer was independent of pH between pH 3.5 and 9.5. However, at pH 2.8 no binding could be observed. Furthermore, if a plate with a previously adsorbed cytochrome *c* layer was immersed in an aqueous

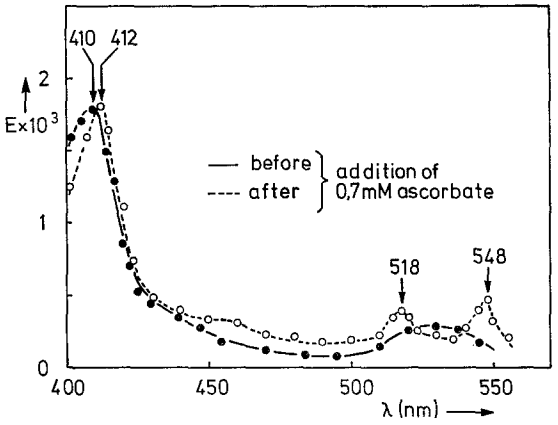

Fig. 6. Spectrum of bound cytochrome *c* in the oxidized and reduced form. The reduction was carried out by addition of 0.7 mM ascorbate (10 mM phosphate buffer, pH 7.6). *E* is the extinction of a single cytochrome *c* layer

solution of pH 2.8, the protein was slowly dissolved. This observation is in agreement with the expectation that below pH 3, most of the phosphatidylinositide molecules are present in the neutral (protonized) form.

The adsorbed cytochrome *c* can be reduced and reoxidized (Fig. 6). For the determination of the absorption spectrum of the bound protein, 5 plates (totalling 10 layers) were used. First, the spectrum of the oxidized form was measured and found to be indistinguishable from the solution spectrum. This was to be expected because the porphyrin ring, which is buried in the crevice of the protein, is relatively well shielded from changes in the environment of the molecule. When the bound ferricytochrome *c* is brought into contact with a 0.7 mM ascorbate solution of pH 7.6, a blue-shift of the Soret band as well as the appearance of new peaks in the vicinity of 520 and 550 nm are observed (Fig. 6). Both changes are characteristic for the formation of reduced cytochrome *c*. However, as the relative absorbances of the Soret band in the reduced and oxidized state are almost equal, whereas for cytochrome *c* in solution the ratio E_{red}/E_{ox} is 1.22, the reduction is possibly not complete. When the ascorbate solution is replaced by an air-saturated buffer solution, the cytochrome is slowly reoxidized. After 4 hr, the spectrum has become completely identical with the original spectrum. It is interesting to note that reduced cytochrome *c* in aqueous solution does *not* react with O_2; however, autoxidability is observed after acetylation of the lysyl residues (*see* Margoliash & Schejter, 1966, pp. 217, 255).

Bilayer Membranes

When a phosphatidylinositide membrane is formed in a cytochrome solution of low ionic strength, cytochrome *c* is bound to the black film The spectrum of the bilayer film with adsorbed protein is shown in Fig. 7 At the peak of the Soret band (410 nm), the mean extinction value from 10 different membranes is $E=(4.0\pm0.2)\cdot10^{-3}$. In principle, the extinction of the black film is caused not only by absorption of light but also by reflection. However, as the reflectivity of the bilayer membrane is only $6\cdot10^{-5}$ (Cherry & Chapman, 1969), the reflection term in E is $6\cdot10^{-5}/2.3$ and may, therefore, be neglected. Thus, $E/2$ gives directly the number, N of cytochrome *c* molecules which are bound to each side of the membrane With $\varepsilon_{410}=106{,}100\ \text{M}^{-1}\,\text{cm}^{-1}$, N is calculated to be $1.1\cdot10^{13}\ \text{cm}^{-2}$ which is in excellent agreement with the value found for a phosphatidylinoside monolayer in the limit of low ionic strength.

The experiment was repeated under the same conditions ($5\cdot10^{-7}$ M cytochrome $c+10^{-3}$ M KCl, pH 7.8) with a film of dioleoyllecithin which has zero net charge. In this case, the extinction at $\lambda=410$ nm was less than $2\cdot10^{-4}$. This means that the binding of cytochrome *c* to the lecithin bilayer is at least 20 times smaller as compared with the phosphatidylinositide membrane.

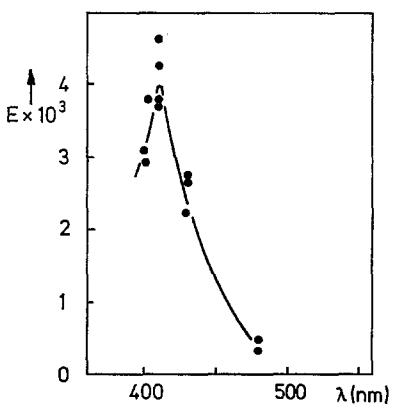

Fig. 7

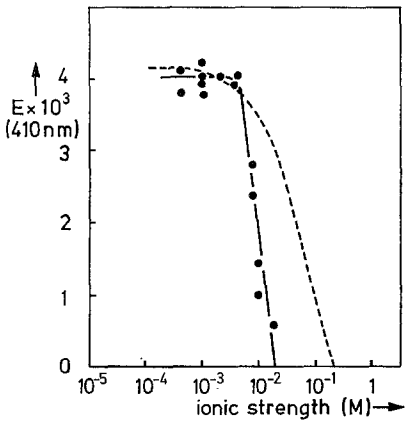

Fig. 8

Fig. 7. Spectrum of a phosphatidylinositide bilayer with bound cytochrome *c*. The membranes were formed in $5\cdot10^{-7}$ M cytochrome $c+10^{-3}$ M KCl (pH 7.8). Each point represents a different membrane

Fig. 8. Binding of cytochrome *c* on a phosphatidylinositide bilayer as a function of the ionic strength (aqueous phase: $6\cdot10^{-7}$ M cytochrome *c* + various amounts of KCl; pH 7.6). Dashed line: binding to phosphatidylinositide monolayers at the same ionic strength (two layers)

The binding of cytochrome *c* to the phosphatidylinositide membrane as a function of ionic strength is shown in Fig. 8. As in the case of the monolayer, the adsorption goes to zero with increasing salt concentration. However, the decrease of E is considerably steeper in the case of the bilayer membrane.

Discussion

Rate of Binding

An upper limit for the rate of binding is given by the kinetics of diffusion-controlled adsorption. The adsorption rate is highest if every protein molecule hitting the surface is bound. In this limiting case, the time course of adsorption may be calculated by solving the diffusion equation under the appropriate boundary conditions. If $N(t)$ is the number of bound protein molecules per cm^2 as a function of time t, n_0 the number of protein molecules per cm^3 in the solution, and D the diffusion coefficient of the protein, then the following relation holds:

$$N(t) = 2\, n_0 \sqrt{\frac{Dt}{\pi}} \tag{1}$$

(*see*, for instance, Frank & v. Mises, 1961; vol. II, p. 555). This equation is valid for sufficiently small times where the area already covered with protein molecules is small compared with the total area. In the derivation of Eq. (1), the influence of the electric field on the diffusion rate has not been taken into account. However, this leads only to a minor correction which is neglected. We may then calculate from Eq. (1) the time τ at which N is equal to, say, one third of the limiting value $N(\infty) \simeq 10^{13}\ cm^{-2}$. With $D = 0.95 \cdot 10^{-6}\ cm^2\ s^{-1}$ (Ehrenberg & Paléus, 1955), we obtain for the three concentrations of Fig. 3 ($c_1 = 10^{-5}$ M, $c_2 = 10^{-6}$ M, $c_3 = 3 \cdot 10^{-7}$ M) the following times: $\tau_1 = 0.25$ s, $\tau_2 = 25$ s, $\tau_3 = 280$ s. The experimental values are: $\tau_1 < 100$ s, $\tau_2 \simeq 110$ s, $\tau_3 \simeq 380$ s. Thus, the observed adsorption times agree, to the order of magnitude, with the minimum times required for diffusion-controlled adsorption. This means that binding occurs almost every time a cytochrome *c* molecule hits the surface. Such behavior would be expected if the interaction is electrostatic. Furthermore, from the fast desorption after a rise of the ionic strength (Fig. 5), we may conclude that penetration of the protein into the lipid layer does not occur to an appreciable extent. However, penetration of cytochrome *c* into lipid monolayers at the air-water interface has been observed by Quinn and Dawson (1969*a*, *b*).

Binding as a Function of Ionic Strength

The experiments described above have shown that the bound cyto chrome *c* layer cannot be removed from the lipid surface by washing witl a pure buffer solution of low ($\leqq 10^{-3}$ M) ionic strength. On the other hand the quick desorption in a more concentrated salt solution indicates that th binding is reversible. We must, therefore, assume that an equilibrium alway exists between bound and dissolved cytochrome *c* at the surface, but tha the equilibrium concentration in the solution is extremely small at lov ionic strengths. The rate of desorption is limited by the diffusion of th protein across the unstirred layer at the surface, which has a thickness o the order of $\delta = 10^{-3}$ cm. If the equilibrium concentration at the surface i less than, say, $c = 10^{-12}$ M, then the diffusional flux would be equal t $cD/\delta \simeq 10^{-11}$ moles/cm^2 s (with a diffusion coefficient of $D = 10^{-6}$ cm^2/s) This means that, of the 10^{13} molecules/cm^2 which are present in the protei layer, less than 1% are desorbed after 10 hr under these circumstances. I principle, the equilibrium concentration may be obtained from the theor of the electrical double layer, if the cytochrome *c* molecule is considere as a charged sphere which interacts with an oppositely charged plan surface at a given ionic strength. The exact theory of this interaction however, is rather complicated because of the special boundary condition and has not yet been worked out (Verwey & Overbeek, 1948; Hull & Kit chener, 1969). Qualitatively, the strong dependence of binding on ioni strength may be understood in the following way. The electrical potentia of the charged surface decays to zero over a distance of the order of th Debye-Hückel length $1/\kappa$ which is a function of the ionic strength J; fo instance, $1/\kappa \simeq 100$ A for $J = 10^{-3}$ M but $1/\kappa \simeq 3$ A for $J = 1$ M. This mean that the total charge of the cytochrome *c* molecule (diameter 31 A) interact with the charged wall at $J = 10^{-3}$ M, but that only a small number of th charged groups contribute to the binding at $J = 1$ M. The calculation of a simplified model based on this picture leads, indeed, to an extremely strong dependence of binding on the ionic strength (Steinemann, 1970).

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