in permeation rates was described (Tsong, 1975).

The observation of membrane potential induced transient pores across phospholipid bilayers should have biological significance. We have shown that the threshold voltage is in a range of about 200 mV for pure DPPC bilayers. In the cell membrane, the threshold potential of lipid domain could be much lower. Thus, as soon as the membrane potential exceeds the threshold potential in a biological membrane, one should consider the lipid layer a poor insulating leaflet, allowing a rapid passage of ions or small molecules.

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Interaction of Cytochrome *c* with Phospholipid Monolayers. Orientation and Penetration of Protein as Functions of the Packing Density of Film, Nature of the Phospholipids, and Ionic Content of the Aqueous Phase†

J. Teissie*

ABSTRACT: Energy-transfer fluorescence quenching has been used to observe the binding of cytochrome *c* to a lipid assembly. The probe (donor), dansylphosphatidylethanolamine, was dispersed either in dipalmitoylphosphatidylcholine, in phosphatidic acid, or in a mixture of the two lipids. The heme of the protein was the acceptor. When the phospholipids were spread in monolayer at the air–water interface, orientation and penetration parameters of the protein relative to the membrane were obtained. The cytochrome is bound with an orientation such that its heme crevice is fully accessible to the aqueous space. Its penetration in the lipid layer is dependent on the ionic content of the subphase and the initial packing of the film. The perturbation induced in the lipid matrix by the binding appears very localized. The same results were obtained with lipid microvesicles. The type of binding of cytochrome *c* to phospholipids observed here implies that there are specific areas on the protein which appear to be different from those involved in its interaction with cytochrome oxidase and other cytochromes. These conclusions are relevant to the existence of different classes of binding sites for cytochrome *c* in the mitochondrial membrane.

In mitochondria, orientation of heme groups of different cytochromes relative to each other and to the membrane are generally accepted to be critical for electron-transfer efficiency. In the case of cytochrome *c*, ESR measurements using labeled species [3-[[(iodomethyl)carbonyl]amino]-2,2,5,5-tetramethylpyrrolidinyl-1-oxy covalently bound on methionine-65] have shown that a specific area is involved in the binding to charged phospholipid microvesicles (Brown & Wuthrich, 1977; Vanderkooi et al., 1973a,b). Our preliminary paper has shown that this is also the case with native cytochrome *c* (Teissie & Baudras, 1977).

A monolayer of phospholipids spread at the air–water interface provides a useful model system for studying lipid–

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protein interactions at the membrane level. The advantages of this kind of study are the defined molecular area, knowledge of the surface pressure, and the known geometry as compared to either microvesicles or BLM. Surface pressure, surface potential, and surface radioactivity have been used to study interactions between proteins and lipids in such a system (Quinn & Dawson, 1969; Khaiat & Miller, 1969; Wooster & Wrigglesworth, 1976; Wiedmer et al., 1978; Popot et al., 1978). It is thus possible to observe the adsorption of a protein from the bulk aqueous phase onto the phospholipid surface and to study the dependence of such a process on the nature of the phospholipid, the packing density of the film, and the ionic content of the aqueous phase. This kind of experiment has been performed by using cytochrome *c* (Quinn & Dawson, 1969, 1970). For low surface pressure the protein was shown to perturb the film drastically (large change in surface pressure), but for high surface pressure a simple adsorption seemed more likely to occur (no change in surface pressure, change in potential and radioactivity). The interactions were shown to be mainly electrostatic. However, with these kinds of studies no information relative to the orientation of the protein was obtained.

Fluorescence is now accepted as a powerful tool for the study of biological membranes (Kasai et al., 1969; Patrick et al., 1971; Mantulin & Pownall, 1977). For protein–lipid interactions when Perrin–Forster energy transfer does occur between a fluorescent emitter embedded in the membrane and a chromophoric acceptor in the protein, not only the binding parameters (Vaz et al., 1977) but also the orientation factor can be obtained (Teissie & Baudras, 1977). The geometrical disposition of the acceptor and donor molecules is critical for the use of such a method, and in this respect monolayers provide a suitable system. We have already reported that fluorescence measurements are feasible on such a system with very low concentrations of the fluorescent probe. Results concerning phase transitions (Teissie et al., 1976), lateral diffusion of lipids (Teissie et al., 1978a), binding of amphiphilies (Teissie, 1979a), and pK shifts of indicators (Teissie et al., 1978b) have already been described.

In the present paper, the fluorescence technique is extended to the study of the interaction of cytochrome *c* with phospholipid monolayers spread at the air–water interface. A fluorescent covalently labeled phospholipid, dansylphosphatidylethanolamine (Waggoner & Stryer, 1970), was chosen as a probe. A mathematical treatment provides a means for determining the penetration into the lipid layer and the orientation of the protein when bound. Conclusions about the organization of the lipid assembly are reported. The same experiments have been performed with microvesicles and compared with our previous results (Teissie & Baudras, 1977) where another probe [not suitable for monolayer (Teissie, 1979a,b)] was used. Here again, orientation and penetration factors for the heme relative to the membrane were determined. The organization of this protein in the mitochondria will be discussed by using these results as evidence for the existence of different kinds of binding sites.

Materials and Methods

Chemicals. Dipalmitoylphosphatidylcholine (P 6138), phosphatidic acid (P 9511) and 12-(9-anthroyl)stearic acid (A 2637) were purchased from Sigma, as was horse heart type III ferricytochrome *c*. Dansylphosphatidylethanolamine was a generous gift of Professor Lussan (Bordeaux, France). Phospholipids were checked for purity by thin-layer chromatography. Salts (Normapur grade, Prolabo, France) were dissolved either in quartz double-distilled water (microvesicle experiments) or in ultra pure water for semiconductor industry (monolayer experiments) (Motorola, France). Salts solutions were filtered through Millipore filters (GSWP and GSTF 04700) to remove any particles. The pH was 5.6 ± 0.2. The temperature was set at 20 ± 1 °C (monolayers) or ± 0.1 °C (microvesicles).

Monolayer and Fluorescence Measurements. Monolayer and fluorescence experiments were carried out on a apparatus which is described elsewhere (Teissie et al., 1976; Teissie, 1979a,b). Fluorescence measurements were obtained by front-face illumination, thereby eliminating the inner filter effect that may occur in the aqueous subphase.

For the binding experiments, a small aliquot of a concentrated solution of protein (500 μM) was injected under the preexistent film through a lateral pipe (Teissie, 1979a), and the subphase was stirred continuously during the entire experiment.

Optical filters used were MTO 3846 for excitation and MTO DH 525 c′ plus Kodak 2E for observation when the probe was dansylphosphatidylethanolamine. For the photobleaching of 12-(9-anthroyl)stearic acid, the set was Schott UG 1 and MTO 10817 (Teissie et al., 1978a) (MTO, France; Kodak, USA; Schott, Federal Republic of Germany).

The electronic time constant was set at 2 s. The data were read by use of a chart recorder.

Microvesicles were obtained by a sonication method (Teissie & Baudras, 1977), using a bath sonicator (Ultrasons, France), and were used within 24 h following their preparation.

Microvesicle Fluorescence. Fluorescence measurements were performed on a FICA Model 55 spectrofluorimeter (corrected) (ARL, France) working at a resolution of 7.5 nm and coupled to a chart recorder; the temperature of the cell (Hellma QS, Germany) was kept constant (±0.1 °C) by use of a circulating bath (Haake FK, Germany).

Diffusion of a Lipid in a Phospholipid Monolayer. Fluorescence recovery after photobleaching of the probe 12-(9-anthroyl)stearic acid was used to report diffusion processes of this labeled lipid (Teissie et al., 1978a).

Determination of Orientation by Energy-Transfer Measurements. Fluorescence quenching through nonradiative energy transfer occurs between a donor and an acceptor and is a function of the separation distance between the two chromophores, the extent of the overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and the relative orientation of the emission moment of the donor and the absorption moment of the acceptor.

The extent of fluorescence quenching of the emission of a probe embedded in a lipid bilayer constitutes a suitable assay for the binding of a protein which contains a suitable chromophore. Cytochrome *c* binding to natural or model membranes was already studied by use of such a method (Vanderkooi et al., 1973b; Vaz et al., 1977; Teissie & Baudras, 1977; Teissie, 1979b).

In an earlier study, we have shown that from the extent of quenching it is possible to determine the relative position of the protein when bound.

Cytochrome *c*, a sphere of radius 1.55 nm (Dickerson et al., 1967), was assumed to be arranged in a continuous monolayer. The transition moment of the heme lies within a diameter plane (Eaton & Hochstrasser, 1967), its application point being at a distance of 1.05 nm from the center of the sphere (Lemberg & Barrett, 1973).

The simulation uses as parameters (Figure 1) $\hat{A}$, orientation of the heme relative to the normal of the membrane (values of $\hat{A}$ larger than 90° are indicative that the heme crevice is

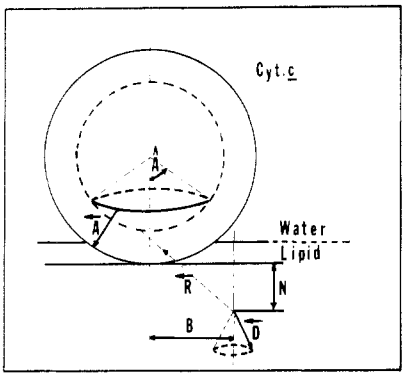

FIGURE 1: Schematic drawing of the relative position of the cytochrome *c* molecule when bound to the lipid layer (monolayer or microvesicle). B is the distance between cytochrome *c* and DPE and *N* is the index of penetration of cytochrome *c* in the film. Both values were determined by fitting the experimental values of limiting fluorescence intensities to the simulated data described under Materials and Methods. $\hat{A}$, the orientation of the heme, was 150.

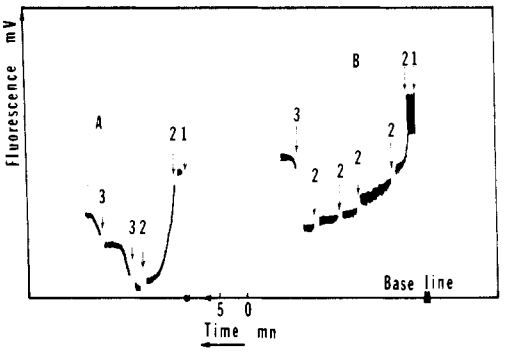

FIGURE 2: Kinetics of the decrease in fluorescence emitted by the probe molecules embedded in a dipalmitoylphosphatidylcholine monolayer when cytochrome *c* molecules are injected under the film. Before the protein injection, the film surface pressure was 4 and 30 $mN \cdot m^{-1}$ for (A) and (B), respectively. The film was spread on pure water. The aqueous phase was continuously stirred. The events are the following: (1) spreading of the film; (2) injection of a concentrated solution of cytochrome *c* into the aqueous phase, giving thus an increment in protein concentration of 0.25 μM; (3) injection of a concentrated solution of NaCl in the aqueous phase, giving thus an increment in salt concentration of 50 mM.

oriented toward the aqueous phase), *B*, distance between the plane of the cytochrome *c* deepest point in the layer and the probe, and *N*, distance between the plane of the cytochrome *c* deepest point in the layer and the plane of the probe, these planes being parallel to the air–water (lipid–water) interface. Small values of *N* are indicative that the protein is deeply buried in the lipid layer.

As previously assumed, the whole cytochrome *c* molecule rotates freely around an axis perpendicular to the interface at the cytochrome *c* deepest point in the layer. Such a model implies the existence of a specific area for the binding of the protein to the lipid array. It was shown that a random binding process giving a value of $K^2 = 2/3$ would not fit the experimental results.

The emission moment of the donor *D* (dansylphosphatidylethanolamine) is distributed on a cone with an axis perpendicular to the interface and a half-angle of 53° (Badley et al., 1973; Teissie, 1979c).

The distance between the application points of both moments, *R*, is calculated from the geometrical description given by Figure 1.

As shown in Figure 1 and described just above, the unit vectors **A**, **D**, and **R** and the distance *R* are explicit function of $\hat{A}$, *N*, *B*, φ_A, and φ_B, where φ_A and φ_B are respectively the azimuthal angles of both cytochrome *c* and the probe. More precisely, we have

$$\mathbf{R} = f_1(\hat{A},B,N,\varphi_A,\varphi_B)$$

$$R = f_2(\hat{A},B,N,\varphi_A,\varphi_B)$$

$$\mathbf{D} = f_3(\varphi_B)$$

$$\mathbf{A} = f_4(\hat{A},\varphi_A)$$

The orientation factor $\kappa = \mathbf{D}\cdot\mathbf{A} - 3(\mathbf{D}\cdot\mathbf{R})(\mathbf{A}\cdot\mathbf{R})$ can be calculated and is also a function of these parameters:

$$\kappa = f_5(\hat{A},B,N,\varphi_A,\varphi_B)$$

The critical distance between the donor and the acceptor, R_0, is computed from

$$R_0 = (J\kappa^2 Q_0 n^{-4})^{1/6} \times 9.79 \times 10^2 \text{ (nm)}$$

where *J*, the spectral overlap integral, was calculated to be 4.6×10^{-14} M^{-1} cm^3 from our experimental data, Q_0, the quantum yield of the unquenched donor, is 0.32 (Letellier, 1973), *n*, the refractive index of the phospholipid layer where the probe is embedded, is chosen to be 1.4 (Steinemann & Lauger, 1971).

As a consequence, we have

$$R_0 = f_6(\hat{A},B,N,\varphi_A,\varphi_B)$$

Taking into account the free rotation of both the cytochrome and the probe around their axis perpendicular to the lipid leaflet, for a given set of values of $(\hat{A},B,N)$, we average our distances R_0^6 and R^6 over the azimuthal angles φ_A and φ_B and obtain $\langle R^6(\hat{A},B,N,)\rangle\varphi_A,\varphi_B$ and $\langle R_0^6(\hat{A},B,N)\rangle\varphi_A,\varphi_B$. The theoretical values of the energy transfer efficiency can be obtained from the Perrin relationship

$$\phi = I/I_0 = \langle R^6\rangle/(\langle R^6\rangle + \langle R_0^6\rangle)$$

where I_0 is the unquenched emission intensity of the donor and *I* its quenched value. Thus ϕ is a function of *A*, *B*, and *N* which can be computed for each set of values of $\hat{A}$, *B*, and *N*.

$$\phi = f_7(\hat{A},B,N)$$

The following boundary conditions were chosen: $\hat{A}$ varies between 0° and 180°; *N* can vary between 0 and 0.75 nm, because the dansylphosphatidylethanolamine is known to have its chromophoric group in the glycerol region when embedded in phospholipid layers (Waggonner & Stryer, 1970); *B* varies between 0 and 2.2 nm, this latter value being obtained when the probe molecule is at the center of a square array of cytochrome *c* molecules (Teissie & Baudras, 1977).

The computed energy-transfer efficiencies were compared to the experimental data and the suitable set of $(\hat{A},B,N)$ was obtained. The listing of the program for the computation of the energy-transfer efficiencies (BASIC) is given in Teissie (1979b).

Results

Binding of Cytochrome c to Dipalmitoylphosphatidylcholine Monolayers. As shown in Figure 2, when an aliquot of a cytochrome *c* solution is injected in the aqueous subphase underneath a DPC monolayer containing DPE (molar ratio 100:1) a decrease of the observed fluorescence is observed. This decrease is faster with increasing amount of cytochrome *c*. The concentration dependence of its magnitude is clear evidence of a saturation process. As already reported (Teissie & Baudras, 1977; Pohl & Teissie, 1975), this decrease is due to an energy transfer between the probe and the heme group.

Table I: Observed Fluorescence at Maximum Binding of Cytochrome c[a]

initial surface pressure ($mN \cdot m^{-1}$)	subphase: pure water	10 mM NaCl	20 mM NaCl	100 mM NaCl
A. Dipalmitoylphosphatidylcholine Monolayers				
4	5 ± 5	30 ± 10	65 ± 10	65 ± 10
30	40 ± 10	59 ± 10	84 ± 10	100
B. Phosphatidic Acid Monolayers				
initial molecular area (nm^2) 0.42		0		0
C. Dipalmitoylphosphatidylcholine–Phosphatidic Acid Mixed Monolayers (10:1)				
initial molecular area (nm^2) 0.42		14 ± 3		75 ± 2

[a] Experimental conditions are described in the text. The fluorescence signal was 100 when no protein was present in the subphase. The probe (DPE) to phospholipid molar ratio was 1:100. Temperature was 20 °C. The fluorescence signal was 100 when no protein was present in the subphase. Then cytochrome c (2 μM final concentration in the aqueous phase) was injected into the well-stirred subphase. After 10 min, the fluorescence signal reached a steady-state value and the signal was then recorded.

Due to the lifetime of a stable signal originating from the film (30 min), the concentration dependence of the kinetics of this process precludes an accurate determination of the binding isotherm in most cases. Nevertheless (except for a film in the gel state on a 0.1 M NaCl subphase), the affinity for the film was strong ($K_{1/2}$ lower than 0.25 μM). For a monolayer in the gel state spread on pure water, the experimental data of the binding isotherm fit a simple equilibrium

$$cyt_{free} + site_{free} \overset{K_d}{\rightleftharpoons} cyt_{bound} \qquad (a)$$

where a "site" is an assembly of phospholipids where the cytochrome c can be anchored.

When the ionic strength in the subphase under the film where cytochrome c was bound is increased by injecting an amount of a concentrated NaCl solution, the quenching is observed to decrease and finally disappear (Figure 2).

The limiting values of the extinction of fluorescence are given in Table I for different initial compression states of the film and ionic content of the subphase. As already described (Quinn & Dawson, 1969), the binding of cytochrome c to lecithin monolayers induces an increase in the surface pressure of the film. Consequently it appears preferable to refer the experiments to their initial compression states, the limiting values of surface pressure being the same (collapse pressure).

When it is assumed that in all experimental conditions a monolayer of bound cytochrome c would have been formed against the film when the protein concentration is increased in the subphase (Pohl & Teissie, 1975), the maximal values of quenching are expected to be directly comparable for the different states of the film and subphase conditions. Within this frame, our data show that the magnitude of the energy transfer depends on both the compression state of the monolayer and the ionic content of the subphase. We have ascertained that the orientation of the probe was unaffected by the binding of cytochrome c by the use of polarized incident light (Teissie, 1979c). These measurements were done with concentrations of protein where such a determination (60% of quenching) was reliable.

No significant change was detected in the lateral diffusion of 12-(9-anthroyl)stearic acid embedded in the DPC monolayer when cytochrome c was present in the subphase, whatever its ionic content. The drastic break in the diffusion coefficients is still present at the end of the liquid-expanded to gel states transition, as already described (Teissie et al., 1978a) (Table II).

Table II: Lateral Diffusion Coefficients of 12-(9-Anthroyl)stearic Acid in Dipalmitoylphosphatidylcholine Monolayers[a]

initial molecular area (nm^2)	0.65	0.45
no cytochrome c in the subphase	11	3.5
0.6 μM cytochrome c in the subphase	8.5	3

[a] Units are 10^{-6} $cm^2 \cdot s^{-1}$. Coefficients were obtained with a fluorescence recovery after photobleaching technique.

Table III: Geometrical Parameters for the Binding of Cytochrome c to Phospholipid Assemblies[a]

subphase	N	B	N	B
A. Dipalmitoylphosphatidylcholine Monolayers[b]				
pure water	0.25		0.5	>1.4
10 mM NaCl	0.5	>1.4	0.75	>1.4
100 mM NaCl	0.5	>1.4	no binding detected	
B. Dipalmitoylphosphatidylcholine–Phosphatidic Acid Mixed Monolayers (10:1)[c]				
10 mM NaCl	0.25	>0.7		
C. Dipalmitoylphosphatidylcholine–Phosphatidic Acid Mixed Microvesicles (10:1)				
10 mM NaCl	0.5	<0.7		

[a] The definitions of B and N are given in the caption of Figure 1. Units are nanometers. Temperature 20 °C. [b] Initial surface pressure 4 $mN \cdot m^{-1}$ for the first set of N and B values and 30 $mN \cdot m^{-1}$ for the second set. [c] Initial surface pressure 25 $mN \cdot m^{-1}$.

Binding of Cytochrome c to Phosphatidic Acid Monolayers. When cytochrome c is injected underneath a monolayer of phosphatidic acid labeled with dansylphosphatidylethanolamine, a rapid decrease of the emitted fluorescence is observed. For all protein concentrations we have used (in the 10^{-7} M range), the limiting value which was observed was a total quenching. This behavior was observed whatever the subphase (Table IB). Under such conditions our simulus calculus cannot be used.

Binding of Cytochrome c to Mixed Films of Dipalmitoylphosphatidylcholine and Phosphatidic Acid (10:1). Injection of cytochrome c under a labeled monolayer gives a rapid decrease of the fluorescence intensity. The velocity of this decrease is intermediate between those observed previously (faster than with the phosphatidylcholine system, slower than with the acidic one). This phenomenon is similar to that reported above for dipalmitoylphosphatidylcholine (concentration-dependent saturation). Data are reported in Table III ($K_{1/2}$ was less than 2.5×10^{-8} M in 10 mM NaCl). As described for the phosphatidylcholine monolayers, the orientation of the probe was unaffected by the binding of cytochrome c to the film.

Binding of Cytochrome c to Phospholipid Microvesicles: Microvesicles of Dipalmitoylphosphatidylcholine. When a suspension of such microvesicles (labeled with dansylphosphatidylethanolamine at a molar ratio 1:250) is incubated with cytochrome c, a spontaneous decrease of the emitted fluorescence is observed of a similar magnitude whatever the ionic strength. The extent of this decrease depends in an exponential way on the protein concentration. When other results relative to the absence of affinity between cytochrome c and bilayered lecithin systems are taken into account (Nicholls, 1974), this observation is to be related to the inner filter effect of the protein on the emission of the probe. These

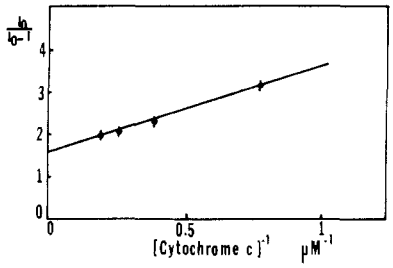

FIGURE 3: Binding of cytochrome *c* to dansylphosphatidylethanolamine-labeled mixed phospholipid microvesicles: double-reciprocal plots of the fluorescence inhibition vs. the protein concentration. Microvesicles were made from a mixture of dipalmitoylphosphatidylcholine and phosphatidic acid (molar ratio 10:1). The probe to phospholipids molar ratio was 1:250. The subphase was 10 mM NaCl. The temperature was 20 °C. I_0 is the fluorescence intensity observed in the absence of added protein and I the fluorescence intensity measured in the presence of protein and corrected for the inner filter effect. Excitation wavelength was 340 nm ($\Delta\lambda$ = 7.6 nm). Identical results were obtained when the observation wavelengths were either 490, 500, 510, or 520 nm ($\Delta\lambda$ = 7.5 nm).

results were used in further studies to calibrate this nonspecific effect when binding occurred. Moreover, the absorption spectrum of cytochrome *c* is known to be affected very little by binding to phospholipid membranes (Vanderkooi et al., 1973b; Steinemann & Lauger, 1971).

Microvesicles of Dipalmitoylphosphatidylcholine and Phosphatidic Acid (Molar Ratio 10:1). The emission spectra of dansylphosphatidylethanolamine embedded in such microvesicles (molar ratio 1:250) is similar to the one observed in the previous system.

When incubated with cytochrome *c*, such labeled microvesicles have their fluorescence instantaneously quenched. The inner filter effect was calibrated by use of pure phosphatidylcholine-labeled microvesicles. Quenching is still observed with these corrected values. Double-reciprocal plots of the decrease of fluorescence intensity (corrected from the inner filter effect) vs. the cytochrome *c* concentration are straight lines (Figure 3). This observation shows that an equilibria occurs between free and bound cytochrome *c*, the latter giving a quenching of fluorescence through an energy-transfer process. As already described (Steinemann & Lauger, 1971), we assumed that when microvesicles were "saturated" with cytochrome *c* the protein was arranged in a continuous monolayer at the surface of the microvesicles, this conformation leading to the extrapolated quenching value. At a temperature of 20 °C in a 10 mM NaCl solution the dissociation constant was found to be $(1.11 \pm 0.07) \times 10^{-6}$ M. This value was obtained whatever the observation wavelengths, this being consistent with the occurrence of an energy-transfer process (Figure 4).

The distribution of phospholipids in microvesicles is known to be asymmetric, the anionic species being concentrated within the inner layer (Berden et al., 1975). The fluorescence properties of this probe have been shown to be pH dependent, the protonated form, present at low pH, being nonfluorescent (Vaz et al., 1978). The diffusion of protons across the bilayer is a slow process as compared with their diffusion in solution (Kano & Fendler, 1978). As a consequence of these two properties, the relative intensities of probe molecules embedded in each layer of the microvesicles can be obtained by decreasing rapidly the pH in the aqueous phase (by adding a small aliquot of 1 N HCl). Thus, it was observed that 78% of the fluorescence was emitted by probe molecules embedded in the outer layer. Energy transfer is a short-range process, and quenching is effective in a range of a few nanometers. As already described (Vanderkooi et al., 1973b; Teissie & Baudras, 1977), only probes embedded in the outer layer are

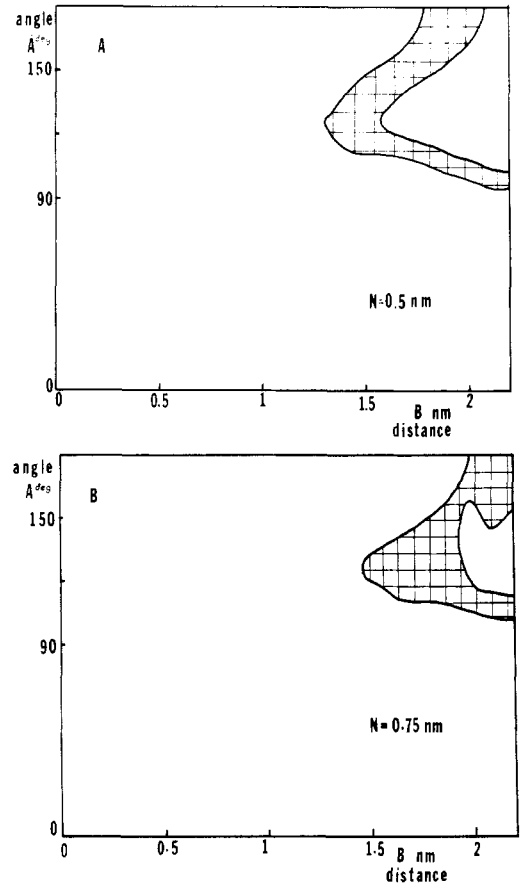

FIGURE 4: Graphic resolution of the orientation–distance relationship for bound cytochrome *c* to a dipalmitoylphosphatidylcholine monolayer (probed by dansylphosphatidylethanolamine). (A) Subphase was pure water; (B) subphase was 10 mM NaCl. The initial surface pressure was 30 mN·m^{-1}. Domains are defined in a (*A*,*B*) space for which, with the value of *N* indicated, a good fit is obtained between experimental and simulated values of quenching. Any couple of values (*A*,*B*) within the hatched area fits the observed value of maximal quenching due to cytochrome *c*. *A* gives the orientation of the heme plane, *B* the distance to the probe, and *N* the penetration in the film (see Figure 1 for an illustration).

affected when cytochrome *c* is bound to microvesicles. Thus it is concluded that the effective quenching is relative to 78% of the fluorescence observed with no protein added and that its limiting value (under "saturating" conditions) is 22%.

Discussion

Cytochrome *c* binding to enzymes and membranes was reviewed by Nicholls (1974). The idea that specific and nonspecific bindings of this protein occur in membranes is supported by much experimental evidence. The complex between the protein and phospholipids is presumed to be stabilized by electrostatic forces, positive charges being present at the surface of cytochrome *c* at physiological pH (Lemberg & Barret, 1973).

Kinetics of the interaction between cytochrome *c* and phospholipid monolayer are functions of (1) the concentration of the protein in the subphase and (2) the density of negative charges on the film. Our observations confirm previous studies (Quinn & Dawson, 1970) where it was shown that the attraction between cytochrome *c* and phospholipid monolayers was a diffusion-limited process and linked mainly to electrostatic interactions. This is particularly clear for dipalmitoylphosphatidylcholine monolayers in a highly compressed state where no binding was detected with a subphase of 0.1 M NaCl. In this case no apolar interactions appear to be involved in the binding.

The simulation procedure linking energy transfer to the geometrical distribution of cytochrome *c* vs. the lipid layer (see Materials and Methods) was applied to our experimental data. For dipalmitoylphosphatidylcholine monolayers, results are displayed in Figure 4. Bound cytochrome *c* has a preferred orientation, with the heme group pointing almost away from the lipid surface ($\hat{A}$ being either 100 or 150°). Such a configuration is obtained whatever the initial state of the film ($\Delta\pi$ = 4 or 30 mN·m^{-1}). The bound protein is progressively buried in the film to the extent that the ionic content of the subphase is decreased. This penetration was larger under similar conditions for a film initially expanded. It should be mentioned that on pure water the orientation was $\hat{A}$ = 80°. As shown in Table IIA and Figure 4, *B* has to be larger than 1.4 nm. This conclusion implies that DPE is close to the middle of an array of bound cytochrome *c* (*B* may be as large as 1.8 nm for an hexagonal array and 2.2 nm for a square array). For a mixed film of dipalmitoylphosphatidylcholine and phosphatidic acid, the same orientation of the heme plane was found. The penetration in the lipid matrix is larger (smaller value of *N*) and again ionic strength dependent. Cytochrome *c* is bound in the very neighborhood of the probe. On mixed phospholipid microvesicles, the orientation of the heme keeps the same value, a small penetration is observed, and again the cytochrome *c* is bound close to the probe. The association of cytochrome *c* to model membranes involves a penetration of the protein in the lipid layer, as described by others (Brown & Wuthrich, 1977; Obratsov et al., 1976), in opposition to the concept of nonpenetration (Van & Griffith, 1975).

All these conclusions were obtained through ESR measurements on bilayered models. For dipalmitoylphosphatidylcholine monolayers, the extent of the penetration was clearly dependent on the ionic content of the subphase and on the molecular packing of the film. The protein was more deeply embedded in the monolayer when the NaCl content was decreased, i.e., when the electrostatic interactions between the film and the cytochromes were increased. Penetration was hindered when the film was tightly packed as compared to a looser state. The occurrence of a penetration of the protein which is modulated by the packing of the lipid matrix provides experimental evidence for the hypothesis postulated from surface-pressure experiments where the increase in surface pressure was postulated to be the consequence of a penetration of the protein in the lipid matrix (Quinn & Dawson, 1969, 1970). When acidic phospholipids were present (pure or mixed with phosphatidylcholine), for identical initial surface pressure (tightly packed films) and same ionic content of the subphase, the penetration of the protein was larger than with the pure neutral lipid. This effect can be due to (at least) two processes. First, electrostatic interactions between charged (positive) groups on cytochrome *c* and the polar group of acidic phospholipids are strong. Thus the protein will preferentially be bound to the charged species when interacting with a mixture. Second, the moiety of the polar head of phosphatic acid is smaller than that of phosphatidylcholine. As the dansyl group has been proved to be at the glycerol level, it will be nearer the water interface in the charged species. This configuration leads to an apparent larger penetration (smaller *N*) of the protein in the film. The discrepancy between the values of *B* found with pure phosphatidylcholine and with mixed phospholipid films can be due to the same phenomenon. Our experiments show that on pure phosphatidylcholine the binding occurs in areas far away from the probe (large values of *B*). In mixed films the binding is to occur on the acidic species as suggested above. When the factors that both the probe and

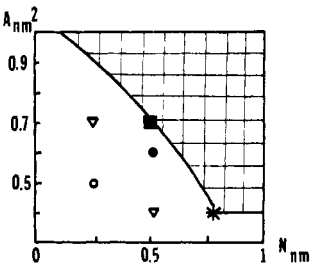

FIGURE 5: Penetration of cytochrome *c* in the lipid under the assumption that proteins and lipids form a mixed monolayer (Figure 6B) as a function of the initial molecular area. The area for each cytochrome *c* molecule (R = 1.55 nm) when bound is $4R^2$. For a penetration of the protein in the phospholipid film whose parameter is N, if P = 0.75 – N (nm), then the area occupied in the film by the protein is $\pi R(2PR - P^2)^{1/2}$. Thus the relative area of the protein is $\pi(2PR - P^2)^{1/2}/4R$. For the mixed film (Figure 6B), the molecular area for each phospholipid when the protein is bound is area(c) = area(0) × [1 – [$\pi(2PR - P^2)^{1/2}$]/4R], where area(c) is the area when the cytochrome *c* is bound and area(0) is the initial area. The total area of the film stays constant, and it is assumed that the phospholipids still form a monolayer as shown in Figure 6B. However, the minimal area for a dipalmitoylphosphatidylcholine molecule is 0.4 nm^2; thus area(c) > 0.4 nm^2. Under such conditions, the values of N which are compatible with the different area(0) values are given in the hatched part. The experimental results are all in the nonhatched area. The mixed film model (Figure 6B) is not in agreement with our results. Experimental values are under the following conditions: dipalmitoylphosphatidylcholine (▽) on pure water, (☆) on 10 mM NaCl, (□) on 0.1 M NaCl; mixture of dipalmitoylphosphatidylcholine and phosphatidic acid (molar ratio 10:1) (○) on 10 mM NaCl (●), as microvesicles in 10 mM NaCl.

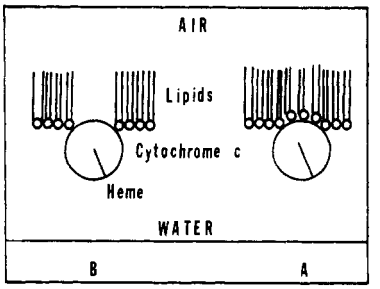

FIGURE 6: Models for the relative position of the protein vs. the monolayer when bound. (A) Protein is bound to a continuous film of lipids; (B) proteins and lipids form a mixed monolayer.

the phosphatidic acid were obtained from egg lecithin where a large degree of unsaturation is present in the hydrocarbon chains and that, on the contrary, dipalmitoylphosphatidylcholine possesses fully saturated chains were taken into account, a phase separation is likely to occur, explaining the observed small value of *B*. In a compressed state the last species is in the gel state when the others are still in the liquid-expanded one; such conditions are highly favorable to a separation of these physically different species. From our experiments it is impossible to distinguish between a prexistent phase separation and a process which was induced by the binding of the protein. A clustering of charged phospholipids when cytochrome *c* is bound to mixed phospholipid microvesicles was observed in ESR studies (Brown & Wuthrich, 1977).

The configuration of the lipids when the protein is bound can be evaluated from the penetration parameter *N* (Figure 5). From our experiments it could be concluded that the configuration which occurs is a continuous film of lipids with bound protein (Figure 6A) and not a mixed film of lipids and proteins. Another argument in favor of such a configuration is the absence of any change in the diffusion coefficients of the photosensitive label embedded in phosphatidylcholine films as a consequence of the binding. This suggests that the changes in the hydrophobic part of the lipid are very subtle,

and this would not be the case for a film where the lipids are expelled by the bound protein.

One of the possible objections to the present study is the conclusion that our simulation fits with two orientations of the heme group for the experimental results. This ambiguity can be removed by reference to our previous study where 8-anilinonaphthalene-1-sulfonate was used as a probe to study the binding of cytochrome *c* to mixed phospholipid microvesicles (Teissie & Baudras, 1977). Only one orientation (A = 150°) is compatible with this previous study.

As reviewed by Nicholls (1974), cytochrome *c* is known to form complexes with proteins in the mitochondria. Studies of the cytochrome oxidase–cytochrome *c* and cytochrome *c* reductase–cytochrome *c* systems (Lemberg & Barret, 1973; Margoliash et al., 1978) show that for such systems the orientation we have just described is not valid though such a configuration leaves free the amino groups (lysine-72, -73) involved in the binding to cytochrome oxidase. Such a discrepancy could be relevant to the existence of "secondary" binding sites as defined by the above reviewer. In mitochondria, cytochrome *c* can be bound (1) only to proteins, (2) to the lipid "annulus" embedding cytochrome oxidase where the properties of the lipids are different from those of the bulk, or (3) to two kinds of binding sites: on another protein or on the lipid matrix as described in this study.

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