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NMR AND ESR STUDIES OF THE INTERACTIONS OF CYTOCHROME *c* WITH MIXED CARDIOLIPIN-PHOSPHATIDYLCHOLINE VESICLES

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Summary

Preparation and structural investigations of 1 : 4 cardiolipin-phosphatidylcholine vesicles with bound ferricytochrome *c* are reported. Size and homogeneity of the vesicles were characterized by column chromatography, ultrafiltration, gel filtration, electron microscopy and ^1H NMR techniques. It was found that the diameter of the vesicles with and without bound cytochrome *c* was approximately 300 Å, that the appearance of the species was typical for single bilayer structures and that the vesicles were stable over several days. ^1H and ^{13}C NMR combined with ESR studies of spin labels covalently bound to cytochrome *c* were then used to investigate structural aspects of these systems. In agreement with earlier studies the present experiments showed that cytochrome *c* is bound on the lipid bilayer surface mainly by ionic interactions. They further provided evidence that binding of cytochrome *c* affects the dynamic behavior of the lipid surface whereas the interior of the bilayer structure is rather insensitive to the protein. As a consequence of cytochrome *c* binding the two kinds of lipids are segregated, with cardiolipin preferentially localized in a surface immobilized boundary layer separating the cytochrome *c* from the fluid bulk of the lipid. Evidence was obtained from the NMR and ESR studies that the bilayer-bound cytochrome *c* has a preferred orientation, with the heme group pointing away from the lipid surface. This indicates some quite striking similarities in the behavior of cytochrome *c* bound to 1 : 4 cardiolipin-phosphatidylcholine vesicles and to intact mitochondria.

Introduction

In recent years it has become increasingly apparent that interactions between protein and lipid are critical to the functioning of biological membranes. However, much of the evidence for the nature of such interactions remains indirect. For instance, changes in enzymatic activity of membrane proteins as a function

of lipid organization [1—3] or changes in transmembrane diffusion dependent on protein [4,5] are indicative of protein-lipid interactions, but provide little insight into the specifics of such interactions. Further understanding of the functional properties of native membranes requires additional information on the structural aspects of protein-lipid interactions.

In classifying the various types of membrane proteins, it has become commonplace to use the categories "extrinsic" and "intrinsic" based on the nature of the conditions which are necessary to remove the protein from the membrane [6]. In such a scheme cytochrome *c* appears to be a typical extrinsic protein since it is removed from the mitochondrial membrane by a variety of mild procedures [7]. On the other hand, cytochrome *c* appears to function in its natural environment as part of a membrane system. Furthermore, in model membrane systems it has been found to exert a variety of effects such as increased lateral membrane pressure [8], enhanced transmembrane diffusion of ions [5] and oxidation of lipids [9]. It thus appears that as an extrinsic membrane protein, cytochrome *c* has significant interactions with lipid membranes which may be important to its physiological function.

In the present paper we have investigated the interactions of cytochrome *c* with single bilayer vesicles composed of a mixture of cardiolipin and phosphatidylcholine with a view to obtaining a more detailed picture of the nature of cytochrome *c*-lipid interactions and protein-lipid interactions in general. Previous investigations of identical preparations have shown that cytochrome *c* functions as an effective catalyst for air oxidation of the lipid membrane [9]. These lipid oxidation processes appeared to occur predominantly in the hydrophobic interior of the membrane [9]. Considering the classification of cytochrome *c* as an extrinsic protein, additional studies on this system were therefore also of interest for an understanding of the structural basis for this catalytic action of cytochrome *c*.

NMR techniques have been applied for the most part in these studies because of the ability of this method to give detailed information about dynamical properties within the membrane system. Furthermore, combined use of paramagnetic species and NMR can lead to spatial information regarding the location and orientation of molecules within lipid systems. In particular, evidence will be presented regarding the location of cytochrome *c* relative to the membrane, effects of cytochrome *c* on lipid dynamic properties at various locations within the membrane, influence of cytochrome *c* on lateral distribution of phospholipid and the orientation of cytochrome *c* relative to the membrane surface.

Materials and Methods

Horse heart cytochrome *c*, cytochrome *c* spin labelled at methionine 65, bovine heart cardiolipin and egg yolk phosphatidylcholine were purchased and/or prepared as previously described [9]. 1 : 4 by weight cardiolipin/phosphatidylcholine vesicles with or without bound cytochrome *c* were prepared in 5 mM Tris/1 mM EDTA buffer at pH 8.0 by previous methods [9]. Preparation of the vesicles and vesicle-cytochrome *c* mixtures were performed under nitrogen to avoid lipid oxidation processes [9]. Previous methods were used for total lipid and protein analyses [9].

The size and homogeneity of the vesicle and vesicle-cytochrome *c* preparations were investigated by electron microscopy and gel filtration. Electron microscopy was performed on a Siemens EM 101 instrument. Copper grids were sprayed with the vesicles in a solution of 1% phosphotungstate which was used as a negative stain. Gel filtration on Sepharose 4B was carried out essentially according to earlier methods [10] except that 5 mM Tris/1 mM EDTA buffer at pH 8.0 was used for elution.

Initial tests for binding of cytochrome *c* to the vesicles were performed by ultrafiltration. An Amicon XM-100 membrane, which was found to pass > 98% of cytochrome *c* and virtually no vesicles was utilized. The effluent from ultrafiltration of the cytochrome *c*-vesicle mixture was analyzed for presence of cytochrome *c* by measurement of the absorption at 410 nm on a Cary 17 spectrophotometer.

Possible changes in cytochrome *c* structure as a result of binding to cardiolipin-phosphatidylcholine vesicles were investigated by ultraviolet-visible absorption spectra taken on a Cary 17 spectrophotometer. For experiments with bound cytochrome *c*, an equal concentration of vesicles without cytochrome *c* was used as the reference to minimize the light scattering effects of the vesicles.

Further characterizations of the vesicle-cytochrome *c* preparations were made with magnetic resonance techniques. ESR measurements were done with a Varian E4 instrument at 9.5 GHz and ambient temperature. Spin label rotational correlation times were estimated according to Stone et al. [11]. For NMR experiments vesicle preparations in deuterated buffer media were used. The p^2H values given for the NMR samples correspond to the pH meter reading without correction for the isotope effect. ^{13}C NMR measurements utilized a Varian XL-100 instrument at 25.16 MHz. ^{13}C T_1 measurements employed the saturation recovery technique [12]. ^1H NMR measurements were made at 100 MHz on a Varian XL-100 instrument and at 360 MHz on a Bruker HXS-360 instrument. ^1H NMR T_1 measurements were performed at 100 MHz using the inversion recovery method [13,14]. T_1 values and upper and lower standard deviations were calculated by fitting the observed peak amplitude versus the delay time τ to a single exponential curve. At least 12 and up to 18 τ values were used for each resonance. The larger of the upper or lower standard deviation was then used to determine a weight factor for calculation of weighted average T_1 and standard deviation values from T_1 measurements on three identically prepared samples. Area measurements for ^{13}C spectra were made by calculating the area of a Lorentzian line with the experimentally determined height and line width. Little or no variation in line width was observed amongst the various samples. ^{13}C and ^1H NMR chemical shifts are referred to external sodium 2,2,3,3-tetradeutero-3-trimethylsilyl-propionate.

Results

(A) Characterization of the 1 : 4 cardiolipin-phosphatidylcholine vesicles with bound cytochrome c

The preparations used for magnetic resonance experiments were prepared by addition of 1 : 100 (mol cytochrome *c*/mol lipid phosphorus) ferricytochrome *c* to 1 : 4 by weight cardiolipin-phosphatidylcholine single bilayer vesicles

which had been pre-formed by sonication. All preparations were made in 5 mM Tris/1 mM EDTA buffer at pH 8.0 and were protected from oxygen during preparation and measurement. As is described in the following, the size and homogeneity of the cardiolipin-phosphatidylcholine vesicles was characterized before and after addition of cytochrome *c* by electron microscopy and gel filtration. Binding of cytochrome *c* to vesicles was examined by ultrafiltration and gel filtration experiments. The structural integrity of cytochrome *c* bound to the vesicles was checked by ultraviolet-visible absorption. Further characterization by ^{1}H NMR will be described in section B.

Binding of the cytochrome *c* to these vesicles was initially tested by ultrafiltration experiments using an Amicon XM-100 membrane which passed cytochrome *c* but not the lipid vesicles. When cytochrome *c* was added to the 1 : 4 cardiolipin-phosphatidylcholine vesicles at 1 : 100 molar ratio, no cytochrome *c* could be detected in the effluent passed through the membrane as measured by visible absorption at 410 nm. At the concentrations used, this implied that less than 0.1% of the total cytochrome *c* was passed through the membrane. By this test ferricytochrome *c* appeared to be strongly bound to the cardiolipin-phosphatidylcholine vesicles. Similar results were obtained with ferrocytochrome *c*.

Electron micrographs of 1 : 4 cardiolipin-phosphatidylcholine vesicles with and without bound cytochrome *c* showed in both cases particles of approx. 300 Å diameter which appeared typical of single bilayer vesicles. Hence, binding of cytochrome *c* at a 1 : 100 ratio (mol. cytochrome *c*/mol lipid phosphorous) did not seem to significantly alter the size of the vesicles. Electron micrographs of the same preparations after 2 days were virtually identical provided that the preparations were protected from oxygen. We have previously described [9] the rapid oxidation catalyzed by cytochrome *c* in these preparations in the presence of oxygen. Such oxidation apparently leads to rapid destruction of the vesicular structure since in the presence of oxygen precipitation of the protein-visicle mixture occurred in a matter of hours. For this reason all experiments were performed under a nitrogen atmosphere.

Fig. 1A shows the elution patterns from Sepharose 4B columns obtained for 1 : 4 cardiolipin-phosphatidylcholine vesicles prepared as described previously [9] and for pure phosphatidylcholine vesicles prepared similarly but sonicated for only 5 min instead of 30 min. For the pure phosphatidylcholine preparation, two peaks elute from the column. The first component (≈ fractions 8—11) elutes at the void volume of the column and has been identified as due to multilamellar lipid particles [10]. The second component (≈ fractions 13—21) has previously been shown to be single bilayer vesicles, with some probable dispersion in size [10]. By comparison with the elution pattern of the pure phosphatidylcholine preparation, the 1 : 4 cardiolipin-phosphatidylcholine preparation elutes almost completely in fractions attributable to single bilayer vesicles. Although the cardiolipin-phosphatidylcholine dispersion was sonicated for a longer time, a process which is known to lead to some destruction of phospholipids [15], subsequent thin-layer chromatography of the lipids after chloroform extraction indicated that destruction of the lipids was minor under the conditions used.

Fig. 1B shows the elution pattern on Sepharose 4B for 1 : 4 cardiolipin-phos-

phatidylcholine vesicles with bound cytochrome *c*. Again virtually all lipid emerges from the column in fractions characteristic of single bilayer vesicles. The elution pattern of the cytochrome *c*-vesicle mixture appears to be slightly

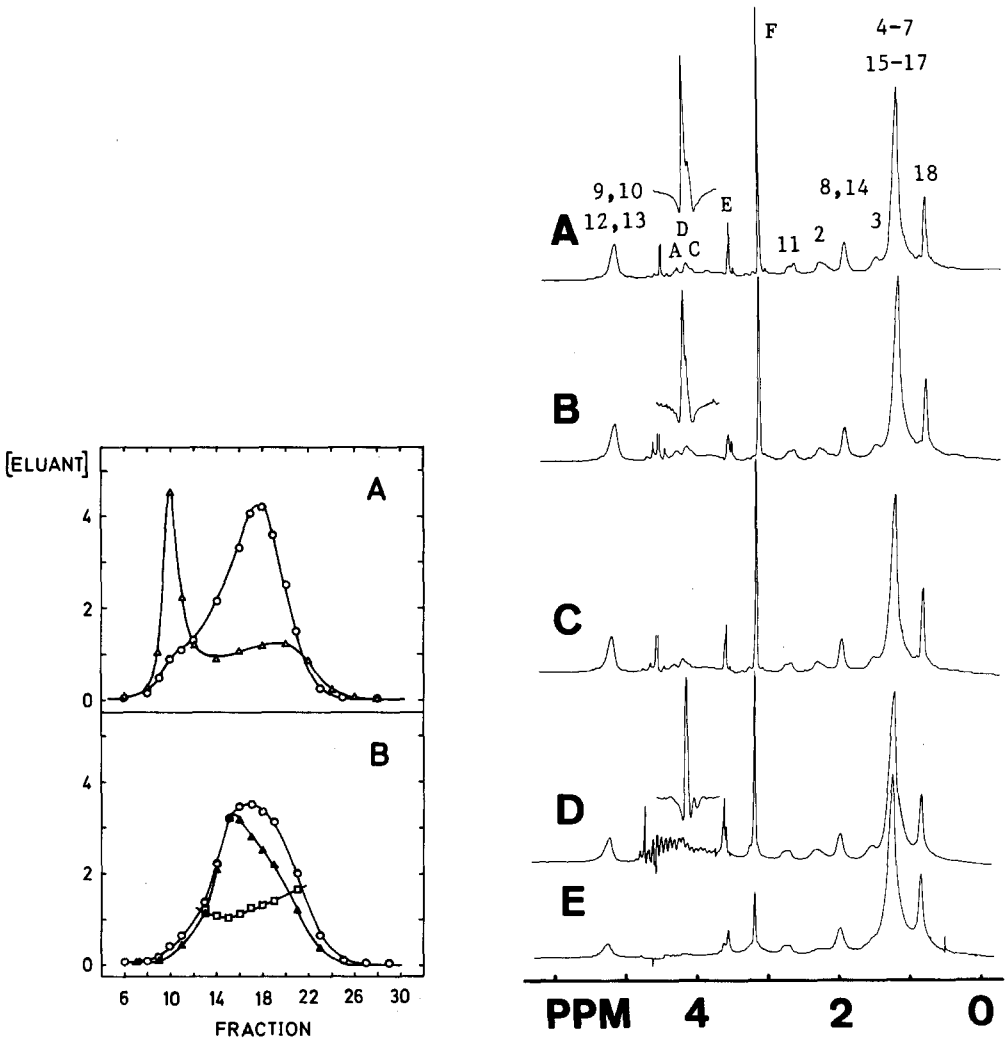


Fig. 1. Column chromatography on a 0.7 X 50 cm column of phosphatidylcholine saturated Sepharose 4B using 5 mM Tris/1 mm EDTA buffer at pH 8.0 as eluent. (A) △, $[PO_4^{3-}] \times 10^3$ for egg phosphatidylcholine sonicated for 5 min; ○, $[PO_4^{3-}] \times 10^3$ for 1 : 4 by weight cardiolipin-phosphatidylcholine sonicated for 30 min. (B) 1 : 4 by weight cardiolipin-phosphatidylcholine sonicated for 30 min, 1 : 100 (mol protein/mol lipid phosphorous) cytochrome *c* added and mixture centrifuged [9]: ○, $[PO_4^{3-}] \times 10^3$; ▲, [cytochrome *c*] X 10^5; □, $[PO_4^{3-}]$/[cytochrome *c*] X 10^2.

Fig. 2. 360 MHz ^1H NMR spectra in a deuterated buffer medium containing 5 mM Tris and 1 mM EDTA at p^2H 8.0 and 25°C of: (A) 1 : 4 cardiolipin- phosphatidycholine vesicles. (B) 1 : 4 cardiolipin-phosphatidylcholine vesicles plus 1 : 100 (mol protein/mol lipid phosphorous) cytochrome *c*. (C) Sample B after standing for 5 days at room temperature under nitrogen. (D) Sample B plus $2 \cdot 10^{-4}$ M Mn^{2+} inside the vesicles. (E) Sample B plus $2 \cdot 10^{-4}$ M Mn^{2+} outside the vesicles. The inserts show the choline *N*-methyl resonance at 3.25 ppm with 4-fold expansion of the ppm scale after resolution enhancement with the sine bell routine [23]. The numbering system used to identify the resonances is shown in the phosphatidylcholine structure in Fig. 3. Chemical shifts are given in Table IV.

broader than that of the pure vesicle preparation, which may indicate a slightly greater size dispersion. This could be due to variation in the amount of cytochrome *c* bound per vesicle since the later fractions from the column showed a slightly greater lipid to protein ratio than the earlier fractions (Fig. 1). These results also support the conclusion that the cytochrome *c* is firmly bound to the 1 : 4 cardiolipin-phosphatidylcholine vesicles. Free cytochrome *c* could be eluted from the Sepharose 4B column only partially and then only with extensive washing of the column. However, when previously bound to 1 : 4 cardiolipin-phosphatidylcholine vesicles, 90% or more of the cytochrome *c* elutes with these vesicles, indicating strong binding of the cytochrome *c* to the vesicles. Extraction of the lipid from the cytochrome *c*-vesicle fractions eluted from the Sepharose 4B column followed by thin-layer chromatography indicated that amongst the various fractions cardiolipin and phosphatidylcholine were essentially equally distributed in the original 1 : 4 ratio. Cytochrome *c*-vesicle samples which had been used for NMR or which had been allowed to stand under nitrogen for several days showed virtually identical elution patterns.

The effect of binding to the lipid vesicles on the structural integrity of cytochrome *c* was investigated by visible absorption. The heme absorption bands of both ferri- and ferrocytochrome *c* appeared to be identical for free and membrane-bound cytochrome *c*. We have further found that the full intensity of the 695 nm band, indicative of the integrity of the iron to sulfur coordination between the heme iron and the sulfur atom of methionine 80 [16], remained observable in our preparations. Previous reports in the literature [7,17] have given conflicting results regarding preservation of this iron to sulfur linkage upon binding cytochrome *c* to various lipid preparations. In agreement with [7], the structural integrity of cytochrome *c*, as tested by the heme group visible absorption, appears to be preserved in our preparations.

(B) ^1H NMR properties of the 1 : 4 cardiolipin-phosphatidylcholine vesicles with bound cytochrome c

The stability and vesicular nature of the 1 : 4 cardiolipin-phosphatidylcholine vesicles following binding of cytochrome *c* has been further investigated using ^1H NMR. Figs. 2A and 2B show 360 MHz ^1H NMR spectra of the vesicle preparation with and without bound cytochrome *c*. Even though cytochrome *c* represents $\approx$ 15% by weight of the protein-lipid mixture, the ^1H NMR spectrum for vesicles with bound cytochrome *c* is virtually identical to that of the vesicles alone. In particular, there do not appear to be any observable protein resonances in the cytochrome *c*-vesicle mixture, even for those protein resonances which are shifted to resonance positions far away from the lipid resonances through interaction with the heme group [18].

The high resolution ^1H NMR spectra of lipid bilayer preparations are highly dependent on the overall size of the lipid structure being observed. Thus, it has been shown that while single bilayer vesicles of 300 Å in diameter give narrow and quite well resolved signals, proton resonances from larger vesicles ($\approx$ 900 Å) are markedly broader and less well resolved, and proton resonances from multilayer dispersions are very broad and largely unresolved [19]. The spectra shown in Figs. 2A and 2B are both typical of small, single bilayer vesicles, indi-

cating that binding of cytochrome *c* causes at most minor changes in vesicle size. This corroborates further the results obtained by electron microscopy and column chromatography as described in the previous section. Fig. 2C shows a spectrum of the same material used for the spectrum in Fig. 2B after standing for 5 days at room temperature under nitrogen. The close similarity of spectra 2B and 2C indicates that over this time period the vesicles are stable in the presence of ferricytochrome *c*. We reemphasize that this is true only so long as the protein-lipid mixture is carefully protected from oxygen during preparation and measurement. In the presence of air, obvious broadening of the ^1H NMR spectrum of the cytochrome *c*-vesicle mixture, probably as a result of vesicle collapse and/or fusion, occurs in a matter of hours.

^1H NMR has also been used to show that the cytochrome *c*-lipid vesicles remain closed with a distinguishable external and internal volume. It has previously been shown for ^1H, ^{13}C and ^{31}P NMR [20—22] that addition of paramagnetic ions to lipid bilayer systems results in broadening or shifting of resonances from nuclei near the bilayer surface. Paramagnetic ions can hence be used to distinguish the internal and external surface of a phospholipid vesicle by selectively adding the paramagnetic ion to the interior or exterior of the vesicle [20—22]. When $2 \cdot 10^{-4}$ M Mn^{2+} was added to the 1 : 4 cardiolipin-phosphatidylcholine vesicles, approx. 65% of the choline *N*-methyl resonance disappeared from the ^1H NMR spectrum with Mn^{2+} only outside the vesicles, approx. 35% disappeared with Mn^{2+} only inside and no resonance was observed with Mn^{2+} both inside and outside the vesicles. As with previous studies [20—22], we interpret this to mean that approx. 65% of the phosphatidylcholine molecules in the vesicle are in the external surface while approx. 35% are located in the internal surface. The two surfaces are distinguishable only if the vesicles are closed and hence impermeable to Mn^{2+}.

Similar results were found for 1 : 4 cardiolipin-phosphatidylcholine vesicles with bound cytochrome *c* as shown in Fig. 2. The inserts in Fig. 2 show the choline *N*-methyl resonance on an expanded ppm scale and after resolution enhancement [23]. For 1 : 4 cardiolipin-phosphatidylcholine vesicles (Fig. 2A), two partially overlapping resonances could be separated by resolution enhancement. The larger peak at lower field and the smaller peak at higher field have previously been assigned [24] to choline *N*-methyl groups in the outer and inner surfaces of the vesicle, respectively. Both the inner and outer choline *N*-methyl peaks are also observable after binding of cytochrome *c* to the vesicles (insert, Fig. 2B). Fig. 2D shows that when Mn^{2+} is added only to the inside of the vesicles with bound cytochrome c, the smaller, high field peak disappears. These vesicles were prepared by sonication of the lipids in the presence of Mn^{2+} followed by addition of cytochrome *c* and ultrafiltration with a dilute EDTA solution. Fig. 2E shows that the larger, low field peak disappears after addition of Mn^{2+} to the outside of these vesicles. Hence the ^1H NMR experiments indicate that the structural integrity of the cardiolipin-phosphatidylcholine vesicles is maintained after binding of ferricytochrome *c*.

To summarize sections A and B, cytochrome *c* can be bound to pre-formed 1 : 4 cardiolipin-phosphatidylcholine vesicles at molar lipid phosphorous to protein ratios of $\approx$ 80 : 1 or greater without destruction or appreciable change in size of the vesicles. For ratios less than $\approx$ 80 : 1, we have found that the pro-

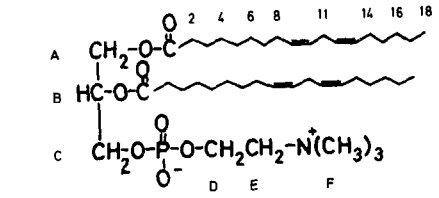
tein-lipid mixture precipitated and hence all the experiments described in this paper have been performed at a lipid phosphorous to protein molar ratio of 100 : 1. At this lipid to protein ratio, vesicles with 25—30 bound cytochrome *c* molecules per vesicle are stable for at least 5 days and continue to be closed and impermeable to metal ions. Stability of the cytochrome *c*-vesicle mixture requires protection from oxygen.

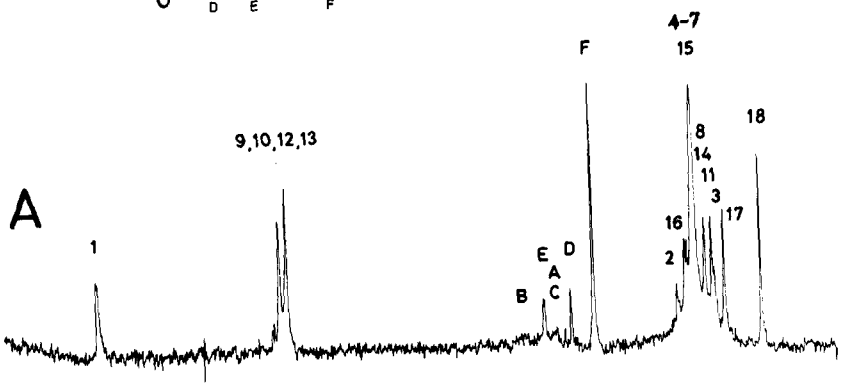
(C) ^{13}C NMR studies of lipid dynamics in 1 : 4 cardiolipin-phosphatidylcholine vesicles with bound cytochrome c

Figs. 3A and 3B show ^{13}C NMR spectra of 1 : 4 cardiolipin-phosphatidylcholine vesicles with and without bound cytochrome *c*. As was found in the ^{1}H NMR spectra (Fig. 2), the ^{13}C NMR spectrum of the protein-vesicle mixture is very similar to that of the vesicles without bound cytochrome *c*. Since 15% by weight of the protein-vesicle mixture consists of cytochrome *c*, some protein resonances should be observable. For example, the concentration of cytochrome *c* α or carbonyl carbons is approximately equivalent to that of the lipid carbonyl carbons, which are observed at 175 ppm. However, no protein resonances are apparent in Fig. 3B. Most probably the protein resonances become very broad as a result of a considerable increase in the rotational correlation time of the cytochrome *c* when it binds to the membrane.

Effects of cytochrome *c* on the dynamic properties of the lipid membrane were manifested in differences between the intensities and spin-lattice relaxation times for corresponding lipid resonances of the 1 : 4 cardiolipin-phosphatidylcholine vesicles with and without bound cytochrome *c*. In particular, evidence was obtained that binding of cytochrome *c* to the vesicles produces two dynamically distinct lipid regions, both of which show altered dynamic features relative to the lipid bilayer in vesicles without bound cytochrome *c*.

It appears to be a common feature of biological membranes that a large proportion of the total material present is not observed in high resolution NMR spectra due to organizational characteristics of the membrane. Various types of interactions, including protein-lipid contacts [25,26], may be the cause of such loss of signal intensity. The spectra of the 1 : 4 cardiolipin-phosphatidylcholine vesicles with and without bound cytochrome *c* have therefore been carefully examined to see if binding of cytochrome *c* causes any changes in the observable intensity of the lipid resonances. Among the resonances where reliable intensity measurements were feasible, i.e. resonances 1; 9,13; 10,12; E; F; 4—7, 15; 17; and 18 in Fig. 3 and Table I, there appeared to be approx. 15% reduction in observable intensity for the carbonyl resonance (resonance 1, Fig. 3) and the main methylene resonance (resonances 4—7, 15, Fig. 3), whereas the other lipid resonances appeared to show essentially the same intensity with or without cytochrome *c* bound to the vesicle (see Table I). These intensity losses do not appear to arise either from slight broadening of these resonances or from changes in nuclear Overhauser enhancement, since virtually no changes in line width or spin-lattice relaxation time could be detected for these resonances. Such intensity losses may arise from lipid which more strongly interacts with cytochrome *c* and hence gives very broad resonances which are not detectable in high resolution spectra. This would be consistent with the expectation that for lipid which has significant interactions with the cytochrome *c*, the mo-





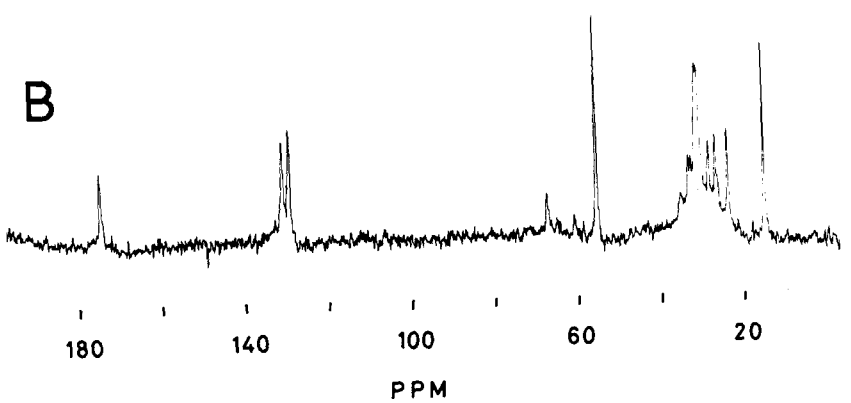


Fig. 3. 25.16 MHz ^{13}C NMR spectra in a deuterated buffer medium containing 5 mM Tris and 1 mM EDTA at p^2H 8.0 and 32°C of: (A) 1 : 4 cardiolipin-phosphatidylcholine vesicles. Total lipid phosphorous concentration 0.23 M; (B) 1 : 4 cardiolipin-phosphatidylcholine vesicles with 1 : 100 (mol protein/mol lipid phosphorous) cytochrome *c* bound. Total lipid phosphorous concentration 0.20 M. The structural formula in the insert shows the numbering system used to identify the resonances. Chemical shifts are given in Table II.

bility of this lipid is strongly influenced by the mobility of the cytochrome *c*, which, as noted above, apparently gives rise to very broad resonances. We note that the intensity losses for the carbonyl and main methylene resonances, but no detectable intensity losses for the resonances from the unsaturated carbons or from the penultimate or terminal carbons of the fatty acid chains, indicates that intensity has primarily been lost from carbon nuclei near the membrane surface. On the other hand, no intensity losses were observed for the choline *N*-

TABLE I

INTENSITY RATIOS FOR THE ^{13}C NMR RESONANCES OF 1 : 4 CARDIOLIPIN-PHOSPHATIDYLCHOLINE VESICLES WITH BOUND CYTOCHROME *c* AS COMPARED TO VESICLES WITHOUT BOUND CYTOCHROME *c*

Samples were measured in a deuterated buffer medium containing 5 mM Tris and 1 mM EDTA at p^2H 8.0 and $T = 32°C$. Areas and standard deviations of resonances relative to the terminal methyl of the fatty acid chain (resonance 18, Fig. 3) were determined from 6 to 15 spectra from three different preparations for vesicles with bound cytochrome *c* (A_c) and for vesicles without bound cytochrome *c* (A_v). Normalization of spectra by determination of total lipid phosphorous in the sample indicated little variation in the area of the terminal methyl resonance between samples.

Resonance (see Fig. 3)	Assignment (refs. 27—29)	A_c/A_v
1	C=O	0.83 ± 0.04
4—7, 15	$(CH_2)_n$	0.86 ± 0.04
9, 13	$CH_2\underline{C}H_2\underline{C}H{=}CH$	0.98 ± 0.05
10, 12	$CH{=}\underline{C}HCH_2\underline{C}H{=}CH$	0.98 ± 0.05
17	$\underline{C}H_2CH_3$	1.00 ± 0.06
18	CH_3	1.0 [a]
E	NCH_2	0.97 ± 0.05
F	$N(CH_3)_3$	0.99 ± 0.03

[a] Assigned value.

methyl resonance from phosphatidylcholine. Possible explanations of these observations are given in Discussion and further evidence for a small proportion of lipid which more strongly interacts with the bound cytochrome *c* is given in section E.

For the observable ^{13}C NMR lipid resonances, information regarding changes in dynamic properties of the lipids as a result of binding cytochrome *c* to the vesicles was obtained by measurement of ^{13}C NMR spin-lattice relaxation times (T_1). In view of the non-observability due to excessive line broadening implicated for lipids in the immediate neighborhood of bound cytochrome *c*, these studies can be expected to primarily give information about long range effects of bound cytochrome *c* on the lipid membrane. The ^{13}C T_1 values measured for vesicles with or without bound cytochrome *c* are shown in Table II. For ^{13}C nuclei with directly bonded hydrogen atoms, which includes all of the lipid carbon nuclei except the carbonyl group, it has previously been shown that spin-lattice relaxation of the ^{13}C nucleus is dominated by dipole-dipole interaction with the directly bonded hydrogen atoms [30]. For isotopic reorientation of the vector joining the hydrogen and carbon nuclei, the spin-lattice relaxation time would then be related to a single correlation time for reorientation of this vector and the number of directly bonded hydrogens. The spin-lattice relaxation time hence provides a measure of rotational mobility. For anisotropic reorientation, which is to be expected for the partially ordered phospholipid molecules in a membrane, T_1 depends on the rates and modes of anisotropic reorientation as well as on the number of directly bonded hydrogen atoms. Thus changes in NT_1, where N is the number of bonded hydrogen atoms, for the ^{13}C nuclei of phospholipid molecules in a membrane can be indicative of changes in the rate and/or mode of reorientation.

A large number of previous studies (e.g. ref. 31—33) have shown that the

TABLE II

^{13}C SPIN-LATTICE RELAXATION TIMES MEASURED IN A DEUTERATED BUFFER MEDIUM CONTAINING 5 mM TRIS AND 1 mM EDTA AT p^2H 8.0 AND T = 32°C FOR 1 : 4 CARDIOLIPIN-PHOSPHATIDYLCHOLINE VESICLES WITHOUT CYTOCHROME *c* (T_{1V}) AND WITH BOUND CYTOCHROME *c* (T_{1C})

N is the number of hydrogen atoms directly bonded to a given carbon atom. T_1 values were calculated as described in Materials and Methods from measurements on three different preparations. The standard deviation indicates reproducibility rather than a unique T_1.

Resonance (see Fig. 3)	Chemical shift (ppm from TSP)	Assignment (refs. 27—29)	NT_{1V} (ms)	NT_{1C} (ms)
1	174.8	C=O	(1850 ± 50) [a]	(1720 ± 55) [a]
2	35.0	$\underline{C}H_2CO_2$	470 ± 50	300 ± 40
3	25.9	$\underline{C}H_2CH_2CO_2$	720 ± 50	700 ± 60
4—7, 15	30.9	$(CH_2)_n$	880 ± 30	900 ± 30
8, 14	28.2	$CH_2\underline{C}H_2CH{=}CH$	1100 ± 40	1080 ± 50
9, 13	130.6	$CH_2CH_2\underline{C}H{=}CH$	545 ± 20	585 ± 20
10, 12	128.8	$CH{=}\underline{C}HCH_2\underline{C}H{=}CH$	595 ± 25	630 ± 25
11	26.5	$CH{=}CH\underline{C}H_2CH{=}CH$	1040 ± 40	1060 ± 40
16	32.9	$\underline{C}H_2CH_2CH_3$	1370 ± 50	1490 ± 50
17	23.5	$\underline{C}H_2CH_3$	2560 ± 45	2800 ± 40
18	14.7	CH_3	7980 ± 100	8640 ± 125
A, C	64.4	CH_2 glycerol	— [b]	— [b]
B	72.0	CH glycerol	— [b]	— [b]
D	60.6	OCH_2 choline	900 ± 70	610 ± 50
E	67.2	NCH_2 choline	640 ± 40	470 ± 30
F	55.0	$N(CH_3)_3$	1410 ± 45	1110 ± 45

[a] For the carbonyl resonance T_{1V} and T_{1C} are given.
[b] T_1 not determined because of insufficient signal : noise ratio for these lines.

mobility and/or ordering of the phospholipid molecules in a membrane is not the same for all parts of the lipid molecule. In particular, it has been shown that the ordering of the phospholipid molecule is greatest near the glycerol backbone and decreases both along the fatty acid chains and along the polar head group [31—33]. This is also reflected in the ^{13}C spin-lattice relaxation times, as has been previously noted [34] and as is apparent from the NT_1 values shown in Table II. The NT_1 values are smallest for nuclei close to the glycerol moiety indicating that this region of the membrane has the lowest mobility and/or highest degree of ordering. The larger NT_1 values for nuclei further along the fatty acid chains or along the polar head group are indicative of greater mobility and/or less order than exists near the glycerol moiety. Table II further shows that the ^{13}C NT_1 values for vesicles with bound cytochrome *c* are also smallest for nuclei near the glycerol moiety and increase for nuclei further internal or external in the membrane. This indicates that the basic pattern of mobility and/or ordering across the lipid membrane is not noticeably altered by binding cytochrome *c* to the vesicles.

Although the basic pattern of mobility across the lipid membrane was maintained after cytochrome *c* was bound, some perturbations in the lipid bilayer are indicated by the measured NT_1 values. Fig. 4 shows plots against position in the membrane of the function:

$$\left(\frac{1}{NT_{1C}} - \frac{1}{NT_{1V}}\right) \Big/ \frac{1}{NT_{1V}} = \frac{T_{1V} - T_{1C}}{T_{1C}}$$

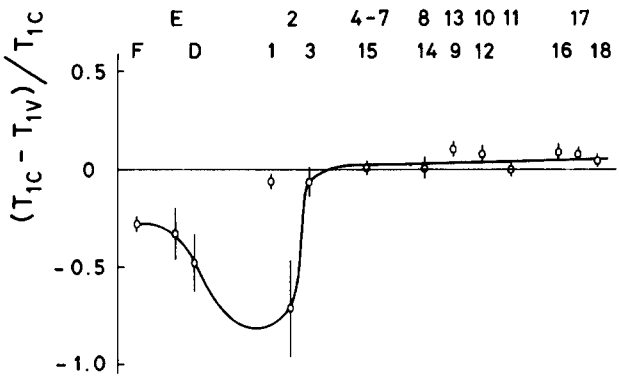


Fig. 4. Plot of change in $1/NT_1$ of ^{13}C versus position in the membrane for the lipid resonances of 1 : 4 cardiolipin-phosphatidylcholine vesicles with 1 : 100 bound cytochrome *c* (NT_{1C}) relative to 1 : 4 cardiolipin-phosphatidylcholine vesicles without cytochrome *c* (NT_{1V}). Note that:

$$\frac{(1/NT_{1V} - 1/NT_{1C})}{1/NT_{1V}} = \frac{(T_{1C} - T_{1V})}{T_{1V}}.$$

Values of $(T_{1C} - T_{1V})/T_{1C}$ correspond to fractional changes in "effective mobility" following binding of cytochrome *c* to the vesicles with negative values indicating decreased mobility (see text). The numbering system corresponds to that shown in Fig. 3 and Table II. Resonance I deviates from the plotted curve since the carbonyl group has no directly bonded hydrogen atoms.

where T_{1V} and T_{1C} are the observed spin-lattice relaxation times for vesicles in the absence and presence of cytochrome *c*, respectively. The physical meaning of this function is readily understood for systems where the carbon-hydrogen dipole relaxation is dominated by isotropic rotational motions in the limiting situation of extreme motional narrowing. In this case, this function would represent a fractional change in the rotational correlation time of the internuclear vector, with negative values corresponding to decreased mobility. In the present anisotropic system, changes in this function may reflect variations in mobility and/or order resulting from binding of cytochrome *c* to the lipid membrane. Fig. 4 shows that this function differs from zero only near the membrane surface with the largest differences near the glycerol moiety and with essentially no differences from zero for nuclei further into the membrane than the second methylene of the fatty acid chain. These results suggest that the bound cytochrome *c* may have a long range effect on the surface of the lipid bilayer. This could be consistent with increased lateral pressure at the lipid bilayer surface as has been found for cytochrome *c* bound to lipid monolayers [8], but the present experiments do not exclude the possibility that this apparent long range effect could actually result from exchange of lipid in contact with the protein. Little or no apparent long range effect was observed in the interior of the membrane, which is consistent with ESR studies on similar systems [35].

(D) ESR studies of spin-labelled cytochrome c interacting with lipids

In this section the ESR characteristics of a nitroxide spin label covalently bound to methionine 65 of cytochrome *c* are described for free cytochrome *c* and for cytochrome *c* bound to lipids. The results of these experiments are compared to earlier work [36,37] on the binding of the spin-labelled cytochrome *c* to mitochondria.

It has previously been shown that covalent binding of a nitroxide spin label

to methionine 65 of cytochrome *c* did not appear to cause any significant change in protein structure and that the spin-labelled cytochrome *c* was fully active in mitochondria [36,37]. In agreement with the earlier reports [36,37], we have found that the bound spin label gives a sharp three line spectrum characteristic of a mobile nitroxide group. The rotational correlation time of approx. $7 \cdot 10^{-10}$ s measured for the spin label is considerably shorter than the correlation time expected for cytochrome *c* on the basis of the Stokes-Einstein equation, i.e. approx. $3 \cdot 10^{-9}$ s. This result was previously suggested to indicate considerable local motion of the spin label independent of overall cytochrome *c* rotation [37]. This would be compatible with the surface location of methionine 65 in the crystal structure of cytochrome *c* [38].

Table III shows the measured rotational correlation time for covalently attached spin label in free cytochrome *c* and in cytochrome *c* bound to a 1 : 4 cardiolipin-phosphatidylcholine mixture either in the form of sonicated vesicles or as a dispersion prepared by previous methods [39]. Table III also shows the effect of increasing sucrose concentration on the spin label correlation time for free, vesicle-bound and dispersion-bound cytochrome *c*. For free spin-labelled cytochrome *c*, the spin label rotational correlation time increases markedly with increasing sucrose concentration. In contrast, while binding of spin-labelled cytochrome *c* to the lipid dispersion caused an increase in spin label rotational correlation time relative to free cytochrome *c*, increasing sucrose concentration did not affect the spin label rotational correlation time for the dispersion-bound cytochrome *c*. Virtually identical results had previously been found when cytochrome *c* spin labelled at methionine 65 was bound to mitochondria [36,37]. The results for mitochondria were previously suggested to indicate that the spin label attached to methionine 65, which lies at the protein surface [38], is located between the protein and the membrane and hence is not influenced by solution viscosity [36,37]. This conclusion involves, as discussed earlier [37], the assumption that the spin label bound to methionine 65 of cytochrome *c* does have local motion relative to the entire cytochrome *c* molecule and that this local motion can be influenced by changes in solution viscosity [40]. If this assumption is correct, the present results suggest that for the 1 : 4 cardiolipin-phosphatidylcholine dispersion, the spin label attached to methio-

TABLE III

ISOTROPIC ROTATIONAL CORRELATION TIME CALCULATED FROM THE ESR SPECTRUM OF A SPIN LABEL BOUND TO METHIONINE 65 OF CYTOCHROME *c* AS A FUNCTION OF SUCROSE CONCENTRATION IN 5 mM TRIS/1 mM EDTA BUFFER AT pH 8.0

The spin-labelled cytochrome *c* was either free (τ_c) or bound to a 1 : 4 cardiolipin-phosphatidylcholine mixture present as a dispersion (τ_d) or as sonicated vesicles (τ_v).

Sucrose concentration (w/v%)	τ_c (s × 10^9)	τ_d (s × 10^9)	τ_v (s × 10^9)
0	0.7	1.0	0.7
30	1.3	1.0	0.7
50	2.1	1.1	0.9
60	3.8	1.0	(1.8) [a]

[a] The increase in τ_v at high sucrose concentration may be due to perturbation of the vesicles (see text).

nine 65 of cytochrome *c* is also located between the protein and the lipid membrane.

The results for spin-labelled cytochrome *c* bound to 1 : 4 cardiolipin-phosphatidylcholine vesicles (Table III) are intermediate to those of free and dispersion-bound cytochrome *c*. Thus, compared to free cytochrome *c*, binding of cytochrome *c* to vesicles did not cause any significant changes in the spin label rotational correlation time. In contrast to free cytochrome *c*, however, considerable sucrose could be added to the solution without influencing the spin label correlation time significantly. These observations would again be consistent with location of the spin label in the solvent protected region between the protein and the vesicle membrane. At the highest sucrose concentration used (Table III), the behaviour of the cytochrome *c*-lipid vesicle preparation differed from that of cytochrome *c* bound to the lipid dispersion in that the spin label rotational correlation time increased with further increase of the sucrose concentration. Even though it could be shown that the cytochrome *c* remained bound to the lipids in the vesicle preparation at all sucrose concentrations used, it cannot be excluded that the increased spin label correlation time is a consequence of perturbations of the overall vesicle structure by the high sucrose concentrations.

The hyperfine coupling constant of the pyrrolidine spin label in 5 mM Tris/1 mM EDTA at pH 8.0, $a = 15.9 \pm 0.2$ G, was unchanged either by covalent binding to methionine 65 of cytochrome *c* or by subsequent binding of the spin-labelled cytochrome *c* to lipids. Since the hyperfine coupling constant is sensitive to the polarity of the environment [41], the spin label was apparently in a polar environment in all cases. Hence, in the lipid-bound cytochrome *c* the spin label is probably near the polar surface of the membrane. This conclusion was also suggested for cytochrome *c* bound to mitochondria on the basis that the hyperfine coupling constant was unchanged by binding [37].

In conclusion, it appears that qualitatively the present model system of cytochrome *c* bound to pure lipid preparations reproduces some of the characteristics of cytochrome *c* bound to mitochondria. Thus, in both cases there is an indication that the methionine 65 region of cytochrome *c* is oriented towards the membrane and shielded from the external solution. Furthermore, in both cases the hyperfine coupling constant for the spin label attached to methionine 65 is unchanged by binding. Additional data which bear on the position of the spin label and the orientation of cytochrome *c* relative to the membrane in the present model lipid system are presented in the following section.

(E) Lipid ^1H NMR spin-lattice relaxation times for 1 : 4 cardiolipin-phosphatidylcholine vesicles with bound spin-labelled cytochrome c

In the experiments described in this section, the paramagnetic effects of a spin label bound covalently to methionine 65 of cytochrome *c* on the spin-lattice relaxation times of the lipid resonances of 1 : 4 cardiolipin-phosphatidylcholine vesicles have been used to obtain information on the location and orientation of bound cytochrome *c* relative to the lipid membrane. ^1H NMR rather than ^{13}C NMR has been employed in these experiments, despite the poorer resolution of resonances from nuclei at various locations in the membrane, because of difficulty in retaining the ESR signal from the spin label over

the considerably longer time necessary for ^{13}C measurements. The ^{1}H NMR spectra also showed small intensity differences between vesicles with or without bound cytochrome *c*, however, due to the poorer spectral resolution, it has not been possible to assign these changes to specific lipid protons.

However, on the basis of the ^{13}C NMR intensity losses, it is to be expected that corresponding intensity losses occur in the ^{1}H NMR spectra as a result of line broadening of the resonances of phospholipid molecules in direct interaction with cytochrome *c*. Hence the paramagnetic effects in the observable resonance lines are primarily for lipid molecules which do not directly interact with cytochrome *c*. This latter point is born out by the results of the following measurements.

As a reference point for the experiments with spin-labelled cytochrome *c*, ^{1}H NMR spin-lattice relaxation times have been measured for 1 : 4 cardiolipin-phosphatidylcholine vesicles with and without bound cytochrome *c*. Table IV shows that binding of cytochrome *c* to the vesicles results in some reductions in the ^{1}H NMR spin-lattice relaxation times of the lipid resonances. The pattern of these changes is similar to that obtained for changes in ^{13}C spin-lattice relaxation times (Fig. 4 and Table II) upon binding cytochrome *c* to the vesicles. Thus, significant changes in ^{1}H NMR T_1 values are again found only for resonances from nuclei near the membrane surface, with the greatest changes near the glycerol moiety. Little or no change is observed for nuclei in the membrane interior.

Table IV also shows that when spin-labelled cytochrome *c* is bound to the cardiolipin-phosphatidylcholine vesicles, a further reduction in the ^{1}H NMR spin-lattice relaxation time, compared to that for vesicles plus cytochrome *c*, is observed for many of the lipid ^{1}H NMR lines. This further reduction in the ^{1}H NMR T_1 values can be attributed to the influence of the paramagnetic spin

TABLE IV

^{1}H SPIN-LATTICE RELAXATION TIMES MEASURED IN A DEUTERATED BUFFER MEDIUM CONTAINING 5 mM TRIS AND 1 mM EDTA AT p^2H 8.0 AND T = 32°C FOR 1 : 4 CARDIOLIPIN-PHOSPHATIDYLCHOLINE VESICLES WITHOUT CYTOCHROME *c* (T_{1V}), WITH BOUND CYTOCHROME *c* (T_{1C}) AND WITH BOUND CYTOCHROME *c* SPIN LABELLED AT METHIONINE 65 (T_{1SLC})

T_1 values were calculated as described in Materials and Methods from measurements on three different preparations. The standard deviation indicates reproducibility rather than a unique T_1.

Resonance (see Fig. 2)	Chemical shift (ppm from TSP)	Assignment (ref. 47)	T_{1V} (ms)	T_{1C} (ms)	T_{1SLC} (ms)
2	2.38	CH_2CO_2	201 ± 8	159 ± 5	113 ± 6
3	1.58	$C\underline{H}_2CH_2CO_2$	—[a]	—[a]	—[a]
4–7, 15–17	1.28	$(CH_2)_n$	447 ± 9	435 ± 9	342 ± 9
8, 14	2.01	$C\underline{H}_2CH{=}CH$	373 ± 8	350 ± 9	261 ± 6
9, 10, 12, 13	5.31	$CH{=}CH$	442 ± 9	446 ± 11	283 ± 7
11	2.82	$C\underline{H}_2(CH{=}CH)_2$	304 ± 7	283 ± 9	206 ± 6
18	0.89	CH_3	642 ± 17	597 ± 12	433 ± 10
A	4.40	CH_2OCO glycerol	—[a]	—[a]	—[a]
C	4.02	CH_2OP glycerol	—[a]	—[a]	—[a]
D	4.29	CH_2OP choline	—[a]	—[a]	—[a]
E	3.68	NCH_2 choline	358 ± 8	308 ± 7	201 ± 5
F	3.25	$N(CH_3)_3$	388 ± 10	351 ± 9	227 ± 6

[a] T_1 not determined because of insufficient resolution of these lines.

label. The paramagnetic contribution to the spin-lattice relaxation rates, $1/T_{1P}$ can be calculated from the relationship $1/T_{1P} = 1/T_{1SLC} - 1/T_{1C}$, where T_{1C} and T_{1SLC} are the measured spin-lattice relaxation times in the presence of cytochrome *c* and spin-labelled cytochrome *c*, respectively. Fig. 5 shows the resultant values of T_{1P} for the various resonances plotted against the position of the corresponding protons within the lipid membrane. From this plot it is clear that not all positions within the lipid membrane are equally affected by the spin label. In particular, the minimum in T_{1P} observed near the first methylene protons of the fatty acid chains (resonance 2, Fig. 5) indicates that the paramagnetic effect from the spin label is greatest for these protons.

As is outlined in Appendix, we have used a previous theoretical treatment [42] to analyze the paramagnetic effects of the spin label on the lipid ^{1}H NMR T_1 values. The model used for this analysis is shown in Fig. 6. where the relevant parameters are defined. The solid curve shown in Fig. 5 is a theoretical

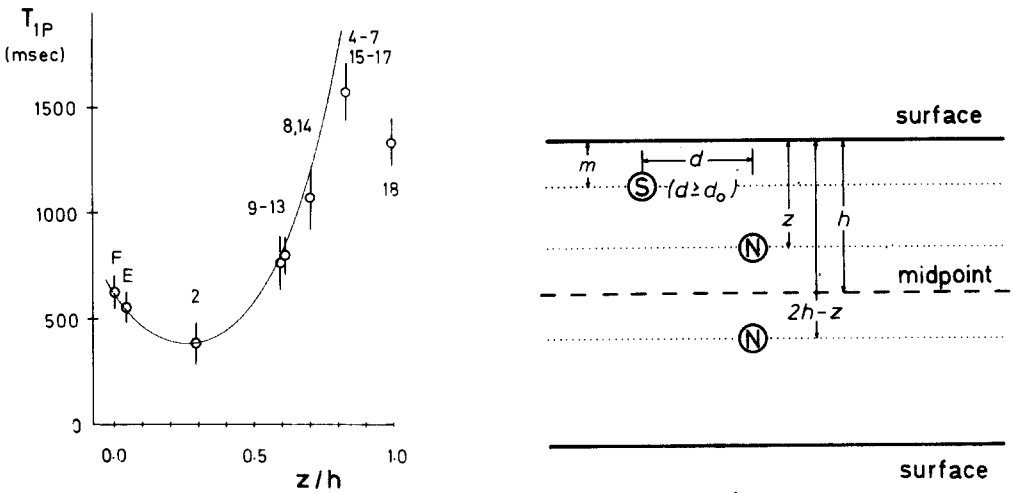


Fig. 5. Plot of T_{1P} versus position in the membrane (z/h, see Fig. 6) for the ^{1}H NMR lines of the lipids of 1 : 4 cardiolipin-phosphatidylcholine vesicles with bound cytochrome *c* covalently spin labelled at methionine 65. T_{1P} is calculated from the relationship $1/T_{1P} = 1/T_{1SLC} - 1/T_{1C}$ where T_{1SLC} and T_{1C} are the spin-lattice relaxation times for the lipid resonances in the presence of bound spin-labelled cytochrome *c* and bound cytochrome *c*, respectively. Note that small values of T_{1P} correspond to greater paramagnetic contribution to the spin-lattice relaxation rates. The solid curve is a plot of Eqn. 2 of Appendix using the parameters $m = 0.26$ and $d_0 = 0.52$. Deviations from the theoretical curve for resonances from nuclei near the middle of the membrane are due to paramagnetic effects on the inner side of the vesicle bilayer (see Appendix).

Fig. 6. Schematic cross section of a lipid bilayer showing the geometric parameters used to calculate the influence of the paramagnetic spin label, S, on the spin-lattice relaxation rate of a given nucleus, N. The spin label and the nucleus are restricted to lateral diffusion in planes parallel to the membrane surface. The geometric parameters *m*, *z* and *h* are normal distances, *m* between the membrane surface and the plane of the spin label, *z* between the membrane surface and the plane of a given nucleus in the same half of the membrane as the spin label, and *h* between the membrane surface and the midpoint of the membrane, i.e. $2h$ is the membrane thickness. The distance *d* represents the separation of the spin label and the nucleus in the plane of the spin label. For nuclei in the same half of the membrane as the spin label, *d* has some minimum value d_0 corresponding to the distance of closest approach of diffusing phospholipid molecules to the spin label. Note that in the NMR experiments the nuclei from both halves of the bilayer are observed as a single resonance and hence the effect of the spin label on both bilayer halves must be considered.

curve based on Eqn. 2 of Appendix. From the parameters used to calculate this curve (see Fig. 5), several conclusions about spatial relationships in the cytochrome *c*-1 : 4 cardiolipin-phosphatidylcholine vesicles can be made. Thus, the minimum in T_{1P} near the first methylene group of the fatty acids (point 2, Fig. 5) indicates that the spin label bound to methionine 65 of cytochrome *c* lies near the plane of the methylene group 2 (see Fig. 3). Assuming that the thickness of the lipid portion of the membrane is 40—50 Å [43—45], the parameters used to calculate the theoretical curve indicate that the spin label is located about 6—7 Å into the outer half of the lipid bilayer. Hence the spin label appears to penetrate slightly into the membrane, implying that the methionine 65 region of cytochrome *c* is indeed in contact with the membrane surface as suggested by the ESR experiments in section D. The assumptions of the model used to interpret these data (Appendix) include that the life-time of the spin label in a plane parallel to the membrane surface is long compared to τ_r and T_{1e}, which are of the order of 10^{-6} s. The spin label-enhanced ^{1}H NMR T_1 values do not exclude rapid rotation of cytochrome *c* about an axis perpendicular to the membrane surface.

Finally, the distance of closest approach of freely diffusing phospholipid molecules to the spin label is estimated to be about 10—12 Å based on a membrane thickness of 40—50 Å. This may be compared to an estimated 6 Å for the closest approach of a lipid molecule to a spin label bound to a second lipid molecule [42]. The greater distance observed here is consistent with a small proportion of lipid which more strongly interacts with the cytochrome *c* and hence prevents the freely diffusing lipid observed in the high resolution NMR spectra from more closely approaching the spin label bound to cytochrome *c*. Such bound lipid was also suggested from the ^{13}C NMR intensity measurements described in section C. Furthermore, it has been possible to directly observe such lipid in ^{31}P NMR experiments (to be published) and the existence of bound lipid would also be compatible with cytochrome *c* induced phase separations observed with ESR techniques [46]. We note further that since cytochrome *c* has a radius of about 17 Å [38], the measured radius of 10—12 Å in which phospholipid more strongly interacts with cytochrome *c* is consistent with binding of cytochrome *c* at the membrane surface with only a limited degree of penetration into the membrane. This radius would also be compatible with the proportion of intensity lost for the carbonyl peak (approx. 15%, section C) and with earlier estimates [39] of the membrane surface area which could be covered by cytochrome *c*, i.e. approx. 15% at the present lipid to protein ratio.

Discussion

Fig. 7 shows a conceptual model, based on the results described in this paper, of cytochrome *c* bound to 1 : 4 cardiolipin-phosphatidylcholine bilayer vesicles. The basic characteristics of this model can be summarized as follows. Cytochrome *c* is bound to the membrane primarily by electrostatic interactions with negatively charged cardiolipin. However, since cytochrome *c* appears to penetrate slightly into the membrane, hydrophobic interactions may also contribute to binding. The binding interactions cause a non-random distribution of

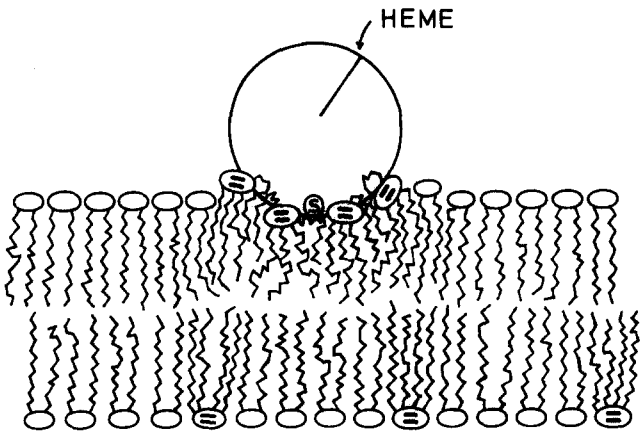


Fig. 7. A conceptual model, based on the present experiments, of the interaction of cytochrome *c* with a cardiolipin-phosphatidylcholine membrane. ○, lecithin; ⊜, cardilipin; ⓢ, spin label attached to methionine 65 of cytochrome *c*. A suggested orientation of the cytochrome *c* heme group relative to the lipid membrane is also shown (see text).

the lipids, with cardiolipin preferentially located in the neighborhood of cytochrome *c*, and also result in a preferred orientation of the bound cytochrome *c*. In the following, several aspects of this model are discussed in more detail.

The present experiments have indicated that binding of cytochrome *c* to 1 : 4 cardiolipin-phosphatidylcholine vesicles produces two classes of NMR behavior amongst the lipids of the membrane. The dynamic properties of lipid in the immediate environment of the cytochrome *c* are strongly influenced by the bound protein at the surface of and slightly into the membrane. This appears to represent a boundary layer of surface immobilized lipid which separates the protein from the bulk of the bilayer structure where the effects on lipid mobility are small and again restricted to atoms near the membrane surface. Earlier ESR spin label studies [35] were not able to distinguish these two lipid classes. However, the earlier ESR studies [35] are compatible with the present results in the sense that in the ESR experiments a surface localized spin label was not utilized and the location of the other spin labels relative to cytochrome *c* was not unequivocal. The present NMR results and the previous ESR results [35] are in full agreement in that both studies indicate that bound cytochrome *c* causes little change in the interior of the lipid bilayer.

The observation of two distinct lipid classes suggests that cytochrome *c* is capable of influencing the lateral distribution of phospholipid molecules in the bilayer membrane. The intensity changes observed in the ^{13}C NMR experiments provide evidence that the changed lateral distribution in the presence of bound cytochrome *c* results from preferential interaction of cytochrome *c* with cardiolipin. Thus, while the intensity losses observed were from nuclei near the membrane surface, no intensity losses were observed for the ^{13}C NMR resonances of the phosphatidylcholine head group. Segregation of cardiolipin in the plane of the lipid bilayer by cytochrome *c* is also consistent with classification of cytochrome *c* as an extrinsic membrane protein. In particular, cytochrome *c* binds more effectively to model membrane systems which include negatively

charged lipids [8] and can be removed from both model membranes [7,8] and the mitochondrial membrane [7] by high ionic strength. We have previously shown that lipid oxidation processes catalyzed by cytochrome *c* are enhanced by inclusion of negatively charged lipids and that even in the presence of negatively charged lipids, such oxidation processes are greatly reduced by addition of salt [9]. Thus, the present observations appear to further corroborate that ionic binding plays an important role in binding cytochrome *c* to lipid membranes. Also, it is not really surprising that positively charged cytochrome *c* should preferentially interact with negatively charged cardiolipin.

In view of the apparent dominant role of ionic binding, it is interesting that the cytochrome *c* appears to have a preferred orientation at the membrane surface. The present ^{13}C NMR experiments indicate that cytochrome *c* is primarily adsorbed at the membrane surface with only a slight degree of penetration into the membrane. On the other hand, the spin label enhanced measurements of ^{1}H NMR spin-lattice relaxation times of the lipids demonstrate that methionine 65 is fixed on or slightly into the lipid membrane, indicating that this side of cytochrome *c* preferentially interacts with the membrane. Based on the X-ray structure of cytochrome *c* [38], it then appears probable that the edge of the heme group accessible at the cytochrome *c* surface is directed away from the lipid membrane as shown in Fig. 7. This preferred orientation of the protein may be related to some extent to the uneven distribution of positive and negative charges on the cytochrome *c* surface [38]. For cytochrome *c* spin labelled at methionine 65, the present ESR experiments on a pure lipid system and earlier studies of intact mitochondria [36,37] yielded rather similar results and provided qualitative evidence that the methionine 65 region of cytochrome *c* lies against the respective membrane. Thus, the preferred orientation of cytochrome *c* determined in the present spin label-enhanced NMR study appears to be similar to that found in intact mitochondria.

Finally, the ability of the NMR method to simultaneously observe all parts of the lipid membrane appears to offer experimental advantages. In particular, comparison of the present experiments with ESR spin label studies on very similar systems [35,46] illustrates the greater resolution of NMR techniques both in the plane of and across the membrane.

Appendix

Brûlet and McConnell [42] have published a detailed theoretical calculation on the influence of a spin label bound to the polar head group of a phospholipid molecule on the lipid ^{13}C and ^{1}H NMR spin-lattice (T_1) and spin-spin (T_2) relaxation times in planar or vesicular phospholipid bilayers. This theory was used to calculate rates of lipid lateral diffusion in phospholipid bilayers, but as pointed out by the authors, can also be used to analyze spatial relationships in membrane systems. The model used for this calculation is shown in Fig. 6, where we have extended the previous model to allow for various positions of the spin label within the membrane and to take into account that a spin label in one half of the bilayer membrane can influence lipid nuclei in the other half of the membrane.

For diffusion rates of the magnitude apparently present in lipid bilayers, i.e.

approx, $1 \cdot 10^{-8}$—$3 \cdot 10^{-8}$ cm^2/s [48,49], and 100 mHz measurements, it can be shown (Hochmann, J., private communication) that the paramagnetic contribution to the lipid spin-lattice relaxation rates from the spin label, T_{1P}, takes a particularly simple form:

$$\frac{1}{T_{1P}} = \frac{3\pi}{20} N\gamma_S^2\gamma_I^2\hbar^2 \cdot f(\omega_I, \tau_c) \cdot \left(\frac{1}{[d_0^2 + (z-m)^2]^2}\right) \quad (1)$$

where N is the number of spin labels per unit membrane area, γ_S and γ_I are the magnetogyric ratios for the unpaired electron and the observed nucleus, respectively, ω_I is the nuclear Larmor frequency and d_o, z and m are the geometric factors shown in Fig. 6. τ_C is given by the relationship $1/\tau_C = 1/T_{1e} + \tau_r$ where T_{1e} is the electron spin-lattice relaxation time for the spin label and τ_r is the rotational correlation time for a phospholipid vesicle. The physical significance of the geometric factor in Eqn. 1 can be understood by noting that $[d^2 + (z - m)^2]^2$ represents the integral over all possible positions in the membrane for a given nucleus relative to the spin label of the usual r^{-6} dependence [50] for dipole-dipole interactions between the unpaired electron and the nucleus observed. From Eqn. 1 it is easily seen that the ratio of the paramagnetic enhancement of the spin-lattice relaxation times for a nucleus with perpendicular distance z relative to a nucleus with distance m, i.e. a nucleus in the same plane as the spin label (see Fig. 6), is given by:

$$\frac{\frac{1}{T_{1P}(m)}}{\frac{1}{T_{1P}(z)}} = \frac{T_{1P}(z)}{T_{1P}(m)} = \left[\frac{d_0^2 + (z-m)^2}{d_0^2}\right]^2 \quad (2)$$

That is, the relative paramagnetic enhancement of the spin-lattice relaxation times for nuclei at various positions in the membrane depends only on geometric factors.

Eqn. 2 takes into account only nuclei in the same side of the bilayer as the spin label, whereas for a given NMR resonance corresponding nuclei from both sides of the bilayer may contribute to the same signal. When both sides of the bilayer are considered, a given type of nucleus has different T_1 values in each side of the bilayer if the spin label is asymmetrically distributed. However, if this difference in T_1 values is not large, the individual exponential decays may not be resolved and a single, average T_1 value is then observed. In this case an average paramagnetic enhancement of spin-lattice relaxation is observed. If the spin label is near the surface of the membrane, the paramagnetic enhancement of relaxation for nuclei in the opposite side of the bilayer membrane will affect the measured T_1 only for nuclei near the middle of the membrane. That is, when the normal distance from the spin label to the observed nucleus in the opposite side of the membrane is sufficiently greater than the normal distance to the corresponding nucleus in the same side of the membrane as the spin label, i.e. $(2h - m - z) > \approx 1.5\ (z - m)$ (Fig. 6), then Eqn. 2 is appropriate.

For the present experiments, Eqn. 2 has been used since the spin-labelled cytochrome *c* is bound to the outside of the vesicle, the spin label is near the vesicle surface and for each resonance only a single, effective T_1 was observed

(Table III). The deviations from the theoretical curve in Fig. 5 for resonances from nuclei near the middle of the membrane arise from paramagnetic effects on nuclei in the inner side of the vesicle membrane.

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