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Interactions of Cytochrome *c* with Phospholipid Membranes

I. Binding of Cytochrome *c* to Phospholipid Liquid Crystals

H. K. KIMELBERG*, C. P. LEE, A. CLAUDE, and E. MRENA

Johnson Research Foundation, School of Medicine,
University of Pennsylvania, Philadelphia, Pennsylvania 19104, and
Laboratoire de Cytologie et de Cancerologie Experimentale,
Université Libre de Bruxelles, Brussels, Belgium

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Summary. Cytochrome *c* added during the formation of lecithin-cardiolipin liquid crystals in 0.015 M KCl is readily bound. After successive washings with 0.15 M KCl, only about 50 % of this bound cytochrome *c* is removed. The remaining cytochrome *c* is resistant to further salt extraction, and the amount of this cytochrome *c* that is bound varies with the concentration of added cytochrome *c* to a maximum binding ratio of 1:70, mole ratio cytochrome *c* to phospholipid. This binding appears to be electrostatic; it is competitively inhibited by increasing the initial molarity of KCl from 0.015 to 0.10 M. Binding of cytochrome *c* is insignificant in the absence of cardiolipin, and is affected by varying the pH. Electron microscope studies of osmium tetroxide-stained thin sections show that the liquid crystals consist of vesicles, each of which contains a large number of concentric, alternating light and dense lines. The dense lines have been identified by other workers with the polar head groups of the phospholipids on the surface of a bilayer, and the light area represents the hydrophobic interior. The addition of cytochrome *c* causes an average decrease in the number of lines per vesicle. It increases the center-to-center distance between two neighboring light or dense lines and the width of the dense lines. On the basis of this evidence and electrostatic binding, it is concluded that cytochrome *c* is binding on the polar surfaces of the phospholipid bilayers comprising the liquid crystalline vesicles.

Complexes of cytochrome *c* with phospholipids have been studied extensively as model systems for protein-lipid interactions and for the reactions of cytochrome *c* in the mitochondria. Two general types of complexes have been described. One type is illustrated by the method used by Das and Crane (1964), in which the cytochrome *c*-phospholipid complex is extracted into isooctane. The structural characteristics of this complex have been recently studied with small-angle X-ray scattering techniques by Shipley, Leslie and Chapman (1969*a*). The other type of complex is not

* Present address: Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York 14203.

extracted from the aqueous phase into isooctane, but is simply centrifuged down as a hydrated complex (Reich & Wainio, 1961; Green & Fleischer, 1963). X-ray scattering methods were used to study the structural characteristics of such complexes by Papahadjopoulos and Miller (1967), Shipley, Leslie and Chapman (1969*b*) and Gulik-Krzywicki, Shechter, Luzzati and Faure (1969). Their findings, as well as results from electron microscopy (Papahadjopoulos & Miller, 1967) indicate that such aqueous complexes consist basically of lamellar units showing a close affinity with the phospholipid liquid crystalline structures or smectic mesophase systems studied in detail by Bangham, Standish and Watkins (1965).

Certain factors have been found to affect the stability of these cytochrome *c*-phospholipid complexes. In the case of the isooctane-extracted complex, increased ionic strength and di- and trivalent cations (Das & Crane, 1964; Das, Haak & Crane, 1965), as well as protamine (Machinist, Das, Crane & Jacobs, 1961), inhibit the formation of the complex. Complex formation is also relatively specific for certain phospholipids (Das & Crane, 1964; Das *et al.*, 1965; Machinist *et al.*, 1961). Thus cardiolipin forms a complex of mole ratio 8:1 lipid to cytochrome *c*, phosphatidyl ethanolamine a ratio of 22:1, and lecithin a ratio of 130:1. Reich and Wainio (1961) found a specificity for the aqueous complex for phosphatidyl ethanolamine, giving a mole ratio of 95:1. Lecithin did not form a visible complex under their conditions; phosphatidyl serine did so only at acid pH.

In this paper, further studies on the binding of cytochrome *c* to phospholipids in the aqueous or liquid crystalline state (Bangham *et al.*, 1965) and factors affecting this binding will be presented. The structural features of these cytochrome *c*-phospholipids complexes are examined by electron microscopy. A preliminary account of some of this work has already been published (Kimelberg & Lee, 1969).

Materials and Methods

Horse heart cytochrome *c* was obtained from Sigma Chemical Co. (Types III and VI). Egg lecithin and bovine cardiolipin were from either the Sylvana Company (Milburn, N.J.) or Supelco, Inc. (Bellefonte, Pa.), and were used without further purification. The homogeneity of the lipid samples was checked by thin layer chromatography on Silica Gel G plates (A. H. Thomas Co.), with a $CHCl_3$:MeOH:H_2O solvent in the proportions 65:25:4. Iodine vapor was used for staining the phospholipids. In the Sylvana lecithin samples, trace quantities of phosphatidyl ethanolamine and sphingomyelin could be detected in the more concentrated spots ($\geqq 50$ µg). Sylvana cardiolipin seemed to contain some lecithin. The lipids were obtained as solutions in either absolute alcohol or chloroform. Samples were stored individually in sealed ampoules under nitrogen at -40 °C, and used only once after opening.

The quantity of lipid present was obtained by dry weight determinations or analysis for phosphate (Barlett, 1959). Triton X-100 and phenazine methosulfate were from Sigma. L-Ascorbic acid was also from Sigma and used as a solution neutralized to the appropriate pH with sodium hydroxide. All other reagents used were of the purest quality available commercially.

Except where noted otherwise, phospholipid liquid crystals containing cytochrome *c* were prepared as follows. Phospholipid liquid crystals or vesicles were prepared essentially according to the method of Bangham *et al.* (1965). Solvent was evaporated from the appropriate mixtures of lecithin and cardiolipin using a stream of nitrogen. The mixtures were held at 35 to 40 °C in a water bath. A cardiolipin-lecithin mixture in the ratio 1:4 by gram weight was used except where noted otherwise. The dried lipids were then swollen in the presence or absence of cytochrome *c* in 10 ml of 0.015 M KCl at room temperature (25 to 28 °C), by mechanical shaking for 1 to 2 min. The pH of the unbuffered suspension was 7.8 to 8.5. The turbid solution was then centrifuged at $30{,}000 \times g$ for 10 min at 4 °C. The pellet was then washed twice with 10 ml of a more concentrated solution of KCl (0.15 M), with resuspension at room temperature and recentrifugation between washes. After the last step, no cytochrome *c* was spectrophotometrically detectable in the supernatant. The pellet was then taken up in a small volume of 0.15 M KCl by a brief homogenization and kept at 0 °C. The pH of the unbuffered pellet was 6.0 to 6.5. Experiments were performed at 25 °C, unless otherwise specified. The concentration of cytochrome *c* in the pellet was determined by reduced-minus-oxidized difference spectra with a split-beam spectrophotometer using the millimolar extinction coefficient $E_{550-540}$ mM^{-1} cm^{-1} reduced minus oxidized of 19 (Margoliash & Frohwirt, 1959). Absolute spectra were obtained by adding an appropriate amount of phospholipid liquid crystals without cytochrome *c* to the reference cuvette to offset light scattering. Kinetic measurements of cytochrome *c* reduction were made with a dual-wavelength recording spectrophotometer.

After the preparative procedure already outlined, specimens for electron microscopy were diluted 3 to 15 times in 0.15 M KCl and centrifuged for 1 min. The resultant pellets were then fixed in a 2% osmium tetroxide solution in 0.1 M phosphate buffer at pH 7.3. After the usual dehydration through graded ethanol, specimens about 1 mm^3 in size were embedded in Epon 812 according to Luft (1961). Thin sections were stained with a 1% aqueous solution of uranyl acetate for 15 to 30 min, and then with lead citrate for 1 to 3 min, according to Reynolds (1963). The stained sections were examined in a Philips electron microscope E.M.-200 calibrated by means of carbon replicas of 28.800 and 54.864 lines/inch gratings.

Results

The initial binding of cytochrome *c* to the phospholipid liquid crystals, and the effect of successive washings in 0.15 M KCl on the amount of cytochrome *c* bound to the centrifuged complex are shown in the Table.

As can be seen, of the 39% of the cytochrome *c* added that is initially bound in 0.015 M KCl, a further 17% is removed by the initial washing in 0.15 M KCl. Successive washings in 0.15 M KCl only removed a further 2 to 3% at each washing. This may well represent fractional loss during recentrifugation, and thus it can be said that the cytochrome *c* remaining after the first wash in 0.15 M KCl is resistant to further salt extraction.

Table. *Effect of successive washings on cytochrome c retention by phospholipid liquid crystals* [a]

Number of washings	KCl wash medium (M)	Cytochrome *c* bound (μmoles)	% bound of added cytochrome *c*
1	0.015	0.21	39
2	0.15	0.12	22
3	0.15	0.11	20
4	0.15	0.09	17
5	0.15	0.08	15

[a] 0.54 μmole cytochrome *c* was added to 8.2 mg lecithin plus 2.3 mg cardiolipin in 10 ml 0.015 M KCl. This was centrifuged at 20,000 × *g* for 10 min, then resuspended with a brief homogenization in 10 ml 0.15 M KCl. After a 0.5-ml sample was removed for spectrophotometric assay, the suspension was centrifuged at 30,000 × *g* for 10 min. The pellet was resuspended in 0.15 M KCl and centrifuged successively according to the Table after samples were removed for assay. Amounts of cytochrome *c* were determined spectrophotometrically as described under Methods.

The binding curve of cytochrome *c* to the phospholipid vesicles for varying cytochrome *c* concentrations, prepared as described in Methods, is shown in Fig. 1 A. The effect of initially swelling the phospholipids and cytochrome *c* in the presence of 0.10 M KCl rather than 0.015 M KCl is also included. It is clear that the binding of cytochrome *c* is decreased at the higher ionic strength, which is consistent with the binding being primarily electrostatic. In support of this, a double reciprocal plot of binding at the two KCl concentrations shows that the inhibition by increased KCl concentration is competitive (Fig. 1 B). Because of the sigmoidal nature of the binding curve at 0.015 M KCl, only the values at higher concentrations of added cytochrome *c* are used to give a linear plot. The maximal binding obtained on a mole basis is 1:70, cytochrome *c* total phospholipid, and an extrapolated maximal binding of 1:32 at infinite concentration of added cytochrome *c*, calculating a molecular weight of 766 for lecithin and 1,382 for cardiolipin and assuming 100% phospholipid.

In agreement with the concept that the interaction between cytochrome *c* and the phospholipids is basically electrostatic between the positively charged cytochrome *c* molecule and the negative groups on the phospholipids, the binding of cytochrome *c* is found to be markedly dependent on the presence of the acidic phospholipid cardiolipin in the cardiolipin-lecithin mixture used here. This is in agreement with the previous results of other workers (Reich & Wainio, 1961; Machinist *et al.*, 1961; Das & Crane, 1964;

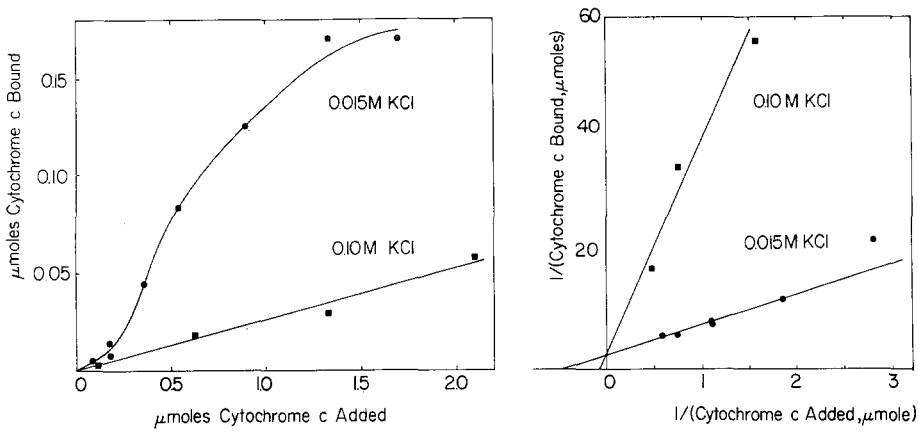

Fig. 1. Binding of cytochrome *c* to mixed cardiolipin-lecithin liquid crystals and the effect of ionic strength. Complexes prepared as described in Materials and Methods. Concentrations of KCl refer to initial swelling medium. These were then washed twice in 0.15 M KCl. Quantities of lipids used were 8 mg lecithin plus 2 mg cardiolipin, finally suspended in 1 ml 0.15 M KCl. B is a double reciprocal plot of A, using the values for concentrations of added cytochrome *c* greater than 0.3 μmole

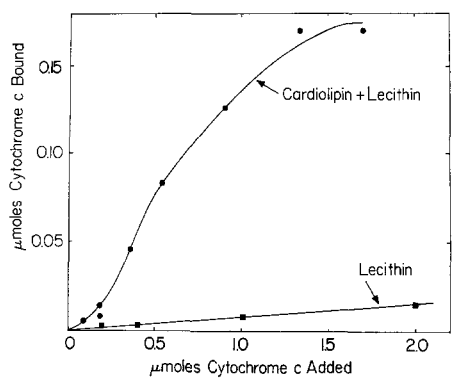

Fig. 2. Comparison of the binding of cytochrome *c* to mixed cardiolipin-lecithin liquid crystals and those containing only lecithin. Conditions as in Fig. 1. Quantities of lipids used were 8 mg lecithin plus 2 mg cardiolipin, or 10 mg lecithin

Das *et al.*, 1965). Lecithin alone binds very little cytochrome *c*. These findings are illustrated in Fig. 2.

The binding of cytochrome *c* is also found to be dependent on pH. This dependency is shown in Fig. 3. The cytochrome *c*-phospholipid complex is first suspended in a dilute solution of an appropriate buffer for the particular pH, as indicated in the figure legend, and then washed twice in the buffer plus 0.15 M KCl. It can be seen that the amount which can be bound is

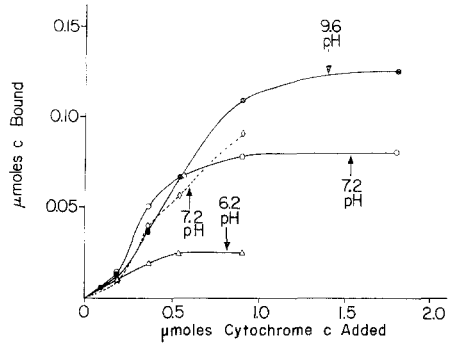

Fig. 3. Effect of pH on the binding of cytochrome *c* to mixed cardiolipin-lecithin liquid crystals. Complexes prepared as described in Materials and Methods. Quantities of lipids used were 8.3 mg lecithin and 2.1 mg cardiolipin, finally suspended in 1.0 ml of the final wash medium. Initial swelling and washing conditions as follows: —▵—▵— Mixture initially swollen in 0.01 M sodium succinate, pH 6.2. Washed twice in 0.15 M KCl, 0.01 M sodium succinate, pH 6.2. —○—○— Swollen in 0.02 M Tris-chloride, pH 7.2. Washed twice in 0.15 M KCl, 0.02 M Tris-chloride, pH 7.2. —•—•— Swollen in 0.02 M Tris-chloride, pH 9.6. Washed twice in 0.15 M KCl, 0.02 M Tris-chloride, pH 9.6. —◇—◇— Swollen in 0.01 M sodium ascorbate, pH 7.2. Washed twice in 0.15 M KCl, 0.01 M sodium ascorbate, pH 7.2

markedly affected. This would be consistent with the idea that the binding is primarily electrostatic. However, the ionizing species responsible for the increase in binding between pH 6.2 and 9.6 is not yet understood. The pKa for the primary phosphate group of cardiolipin could be expected to be around 3, and the isoelectric point for cytochrome *c* is 10.65 (Theorell & Åkesson, 1941). The dotted curve shows the binding of reduced cytochrome *c* at pH 7.2. The cytochrome *c* is maintained reduced by swelling in 0.01 M sodium ascorbate and washing in 0.01 M sodium ascorbate plus 0.15 M KCl. At lower pH values, the centrifuged complex was more compact than at the more alkaline values.

The structural aspects of these mixed phospholipid-cytochrome *c* complexes were examined by electron microscope studies of osmium tetroxide-stained thin sections. Mixtures of lecithin and cardiolipin swollen in both the presence and absence of cytochrome were investigated. Electron micrographs of the preparations without cytochrome *c* are shown in a low-power view in Fig. 4 and at a higher magnification in Fig. 5. It is clear that structures with considerable lamellar structure of alternating light and dense lines are obtained, as described in detail by others (Stoeckenius, Schulman & Prince, 1960; Bangham *et al.*, 1965; Papahadjopoulos & Miller, 1967). One light line plus each half of the dense lines on either side is presumed to correspond to a lipid bilayer (Stoeckenius *et al.*, 1960) and the number of

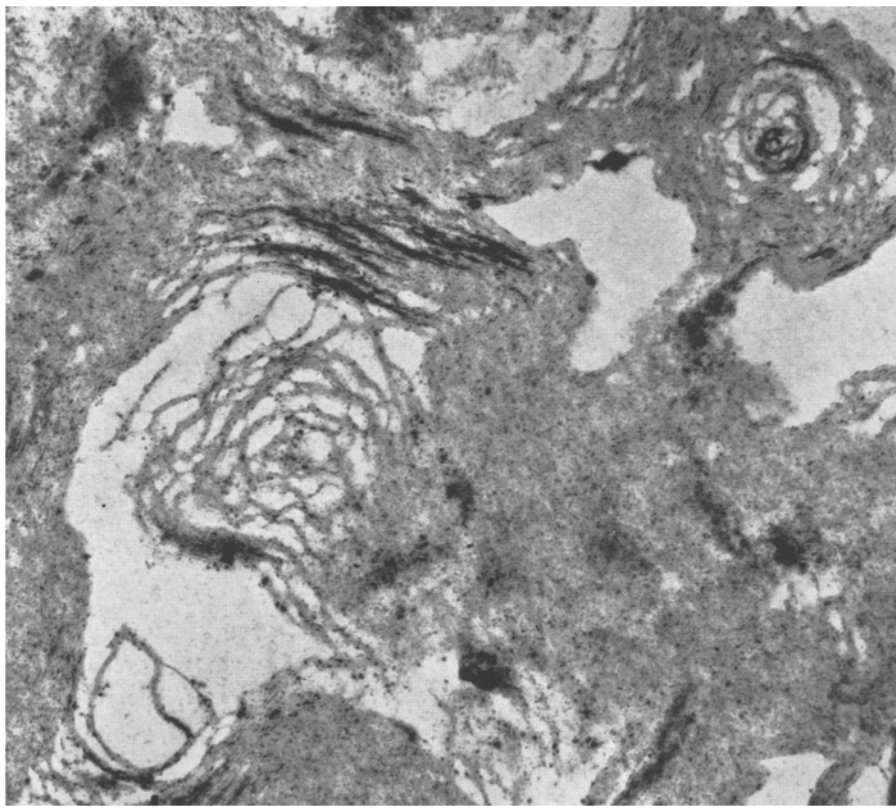

Fig. 4. Low-power magnification electron micrograph of mixed cardiolipin-lecithin liquid crystals in the absence of cytochrome *c*. The quantities of lipids used were 8 mg lecithin and 2 mg cardiolipin. Liquid crystals were prepared as described in Materials and Methods. These were finally suspended in 3 ml 0.15 M KCl, sedimented briefly and fixed in 2% OsO_4 in 0.1 M PO_4 buffer at pH 7.3. The samples were dehydrated in graded ethanol and propylene oxide, and embedded in Epon 812. Thin sections were stained with 1 % uranyl acetate in water for 30 min and then lead citrate for 1 to 3 min. Sections were then examined in a calibrated Philips EM-200 electron microscope. Micrograph taken at 20,000 magnification, enlarged to 40,000

such lamellae in one spherical body can clearly be considerable. The dense lines have been tentatively identified with the polar head groups on the surface of a phospholipid bilayer and the light lines would correspond to the hydrophobic interior occupied by the fatty acid chains (Stoeckenius *et al.*, 1960).

The effect of swelling these phospholipid mixtures in the presence of cytochrome *c* is illustrated in Figs. 6 (low power) and 7, 8a and b (high-power magnification). The amount of cytochrome *c* bound to the phospholipids was 0.14 μmole cytochrome *c*/8 mg lecithin *plus* 2 mg cardiolipin. On a

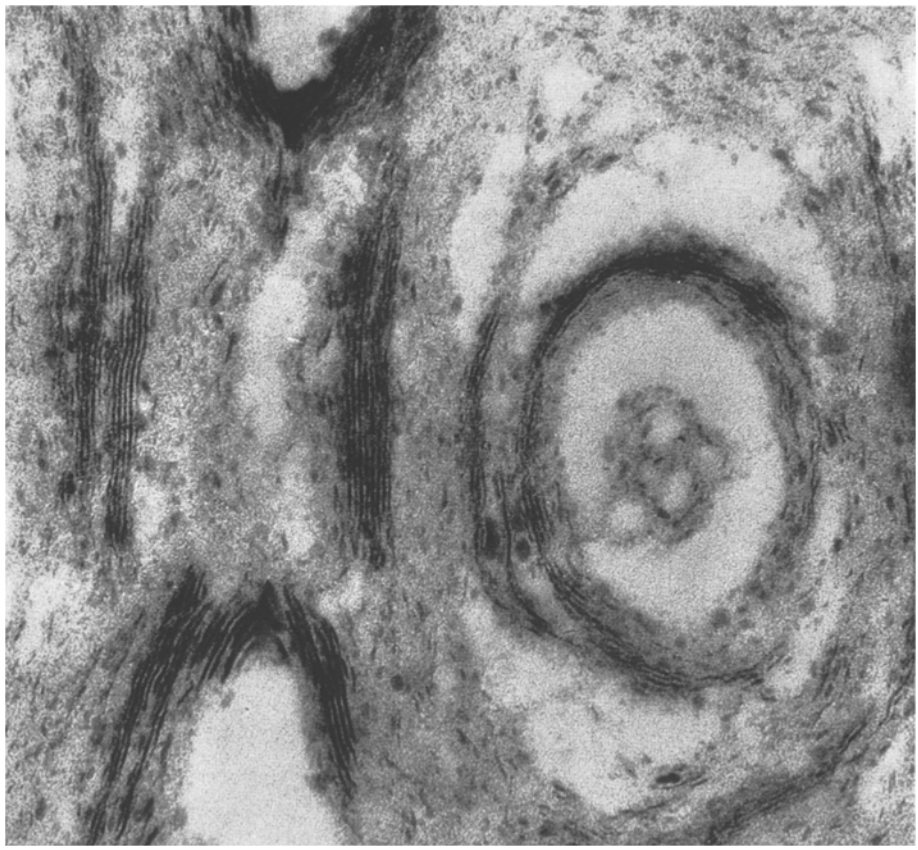

Fig. 5. High-power magnification electron micrograph of mixed cardiolipin-lecithin liquid crystals in the absence of cytochrome *c*. Conditions as in Fig. 4. Micrograph taken at 86,500 magnification, enlarged to 173,000

weight basis, this would be 1.7 mg cytochrome *c*/10 mg total lipid. The effect of cytochrome *c* on the phospholipid liquid crystal structures compared to phospholipids alone may be summarized as follows:

1) Cytochrome *c* is found to increase the orderliness of the lamellae, which show increased stability and cohesion. This may be primarily due to the presence of the protein protecting the phospholipids from being dissolved during the ethanol dehydration, in the preparation of the samples for electron microscopy. Thus the electron micrographs of the phospholipid vesicles in the absence of cytochrome *c* (Figs. 4 & 5) show considerable disruption of structure. The darker areas in these figures show the lamellar structure most clearly and are presumably the areas where no disorganization has taken place.

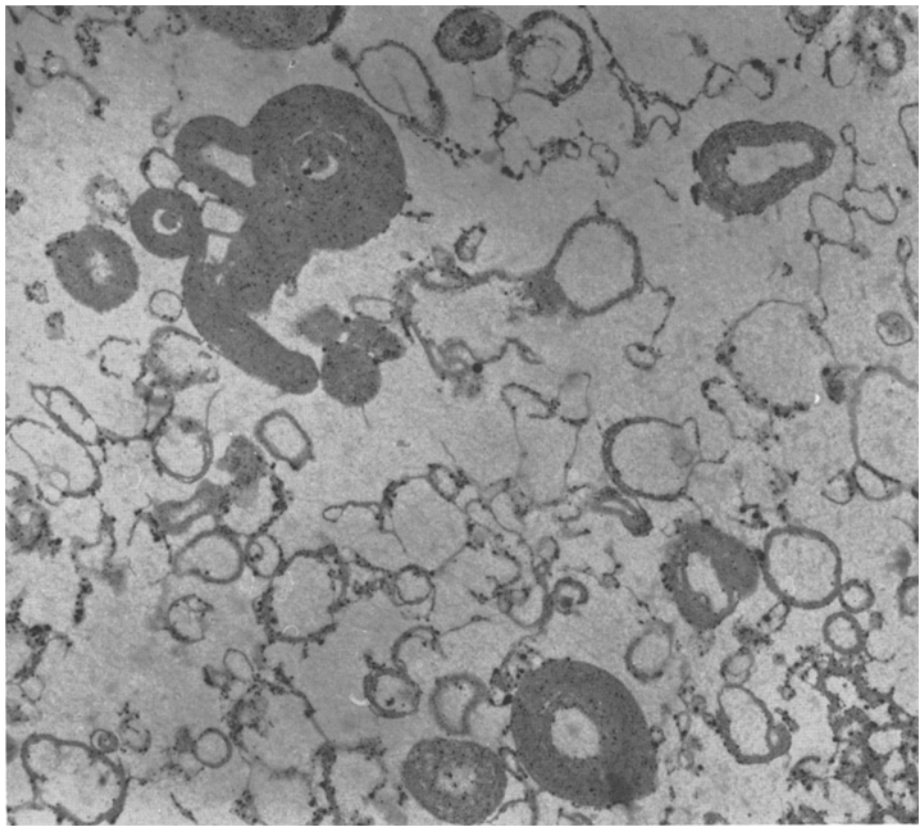

Fig. 6. Low-power electron micrograph of mixed cardiolipin-lecithin liquid crystals in the presence of cytochrome *c*. Final concentration was 0.14 μmole cytochrome *c*/8 mg lecithin plus 2 mg cardiolipin in 15 ml 0.15 M KCl. Sedimented, fixed and stained as in Fig. 4. Micrographs taken at 12,000 magnification, enlarged to 24,000

2) A larger number of smaller vesicles containing only one or several lamellae are evident (*see* Fig. 8a & b). Apparently cytochrome *c* tends to decrease the overall size of the vesicles, in agreement with the results of previous workers (Papahadjopoulos & Miller, 1967).

3) Upon examination at high magnification (>240,000), the thickness of the lamellae appears increased. The width from center to center of the repeating units (i.e., the center-to-center distance between two neighboring dense or light lines) gives a mean value of 88 A for the lamellae containing cytochrome *c* and 45 A in the absence of cytochrome *c*. As can be seen from a comparison of Figs. 5 and 7, the presence of cytochrome *c* seems to increase the width of the dense lines. The small dark bodies seen in Figs. 6, 7, and 8a and b are not specifically associated with the presence of cytochrome *c* as

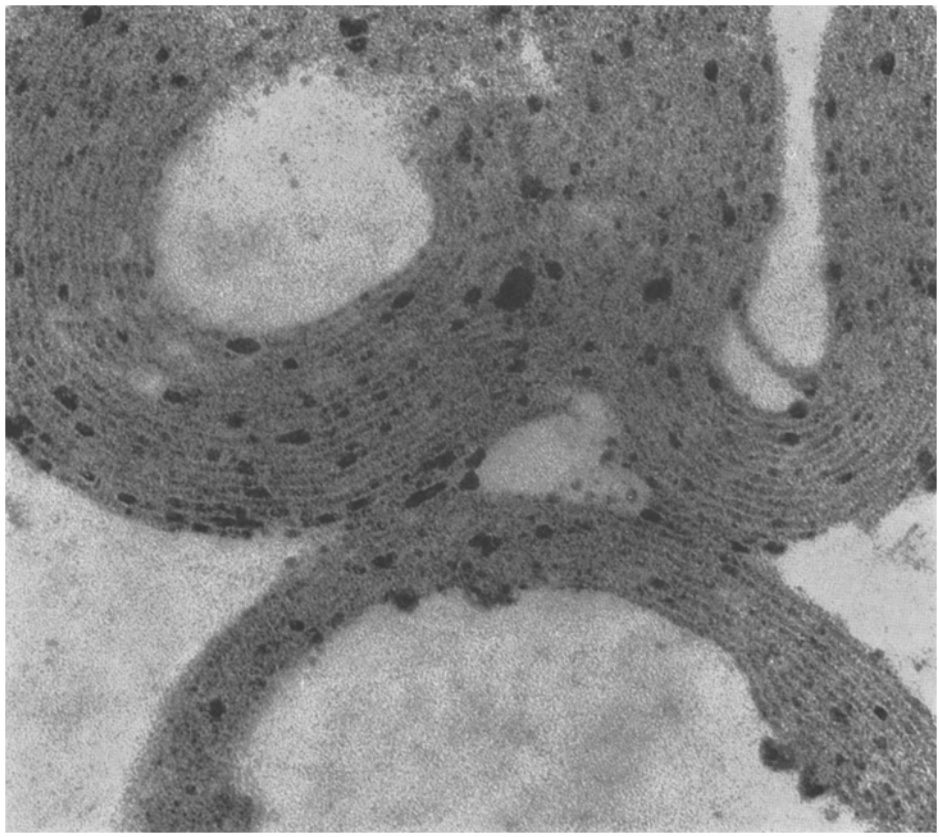

Fig. 7. High-power electron micrograph of mixed cardiolipin-lecithin liquid crystals in the presence of cytochrome *c*. Conditions as in Fig. 6. Micrograph taken at 86,500 magnification, enlarged to 173,000

they can also be seen in Figs. 4 and 5, which represent phospholipid vesicles without cytochrome *c*. The origin of these bodies is not yet clear and can only be resolved by further investigation.

Discussion

It has been suggested from experimental and theoretical considerations (Bangham, 1968) that dry phospholipids in the presence of an aqueous solution of electrolyte form structures consisting of a series of concentric bimolecular membranes which show liquid crystalline characteristics. We have attempted to enclose cytochrome *c* specifically within these membranes by swelling the dry phospholipids, in the presence of cytochrome *c*, in a low

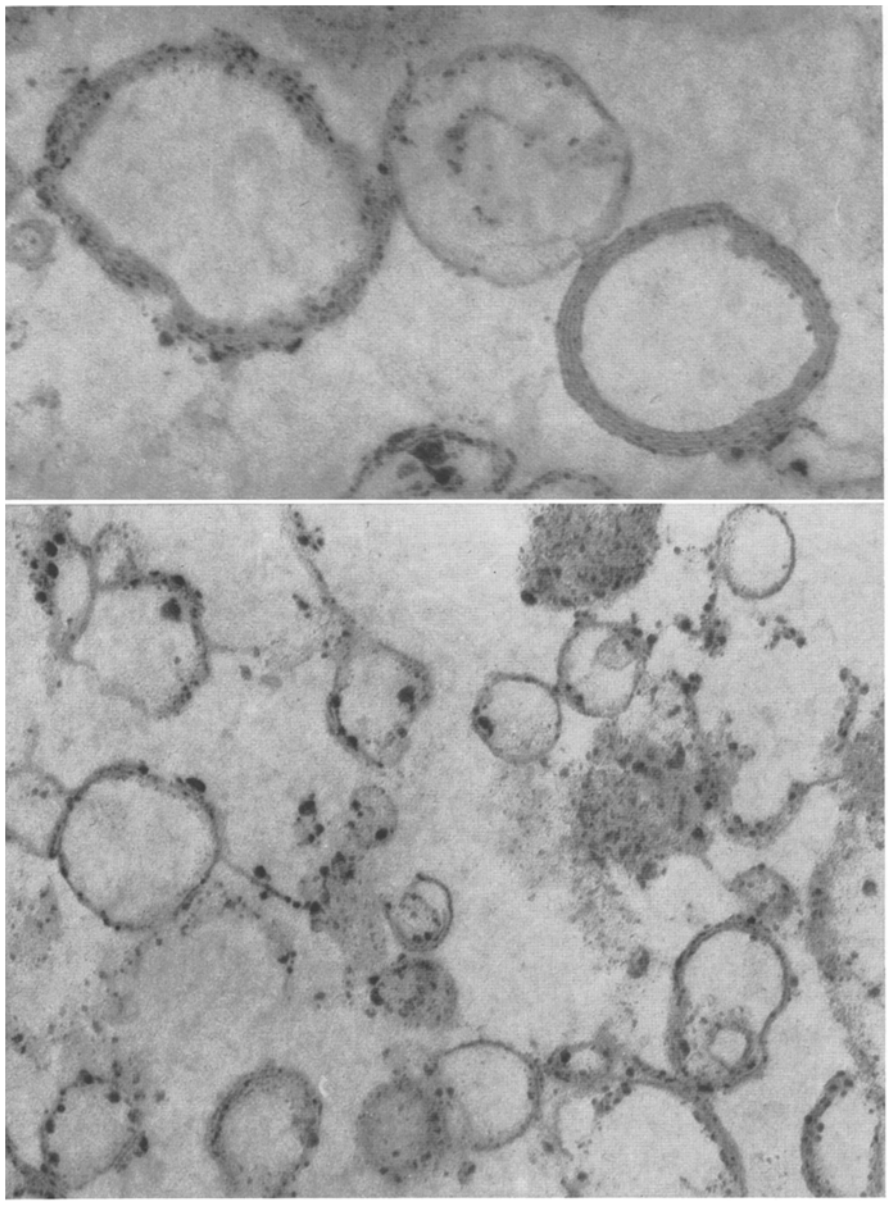

Fig. 8. Electron micrographs of mixed cardiolipin-lecithin crystals in the presence of cytochrome *c*. Conditions as in Fig. 6 and 7. Micrographs taken at 36,000 magnification: *a* enlarged to 80,000, *b* enlarged to 73,000

ionic strength solution. The cytochrome *c* bound on the outside of the outermost membrane is then removed by subsequent washing in a high ionic strength medium. A remaining portion of the cytochrome *c* is then found to be

resistant to further removal by washing (*see* Table), which is likely to be due to separation from the bulk solution by closed membrane barriers.

The cytochrome *c* that is resistant to removal by further washing resembles, in this regard, the endogenous cytochrome *c* of Keilin-Hartree submitochondrial particles (Tsou, 1952), the endogenous cytochrome *c* of submitochondrial particles prepared by sonication (Lenaz & MacLennan, 1966) and the soluble cytochrome *c* trapped inside sonicated submitochondrial particles (Lee & Carlson, 1968). One can envisage cytochrome *c* trapped inside a membrane barrier in these biological systems, in a manner analogous to the scheme outlined above for the phospholipid liquid crystals. In order to account for the fact that endogenous cytochrome *c* is readily salt-extracted from mitochondria after swelling in hypotonic solution (Schneider, Claude & Hogeboom, 1948; Jacobs & Sanadi, 1960), Lenaz & MacLennan (1966) suggested that the mitochondrial inner membrane was only permeable to cytochrome *c* in one direction. A particle in which the inner membrane shows reverse polarity may be produced by sonication (Lee & Ernster, 1966), thus causing a permeability barrier to cytochrome *c* extraction. Since cytochrome *c* in mitochondria can be readily and reversibly salt-extracted (Schneider *et al.*, 1948; Jacobs & Sanadi, 1960), it may normally bind electrostatically on the outside of the inner membrane surface, on or near enough to cytochrome oxidase to permit electron transfer (Nicholls, Mochan & Kimelberg, 1969). In this case, sonication with inner membrane inversion would physically trap cytochrome *c* inside a semipermeable membrane barrier, analogous to the scheme proposed for the phospholipid liquid crystals.

Lecithin is a zwitterion at pH values between 3.5 and 11.5, as shown from monolayer titrations (Papahadjopoulos, 1968). The failure of cytochrome *c* to bind lecithin to any appreciable extent (*see* Fig. 2) is thus understandable if the binding is electrostatic. Cardiolipin, however, has two ionizable phosphate groups which can provide negatively charged sites for electrostatic interaction with the positively charged cytochrome *c* (Theorell & Åkesson, 1941). This specificity for acidic phospholipids is similar to that found in isooctane-extracted complexes (Das & Crane, 1964; Das *et al.*, 1965). Cardiolipin is almost exclusively found in the mitochondrial inner membrane. In the case of guinea pig liver, cardiolipin represents 21.5% of the total lipid, lecithin 44.5% and phosphotidyl ethanolamine 27.7% (Parsons, Williams, Thompson, Wilson & Chance, 1967). Although complex formation in this study is dependent on the presence of cardiolipin in a cardiolipin-lecithin mixture, phosphatidyl ethanolamine also forms a complex with cytochrome *c* (Reich & Wainio, 1961; Das & Crane, 1964),

as do other acidic phospholipids. The extrapolated maximal mole-binding ratio of 1:32 cytochrome *c*/phospholipid (Fig. 1 B) gives a ratio of 1:4 in terms of cardiolipin alone. This is similar to the ratio found for the isooctane-extracted complex (Das & Crane, 1964).

The evidence presented in this paper on the inhibition of the initial binding by increased ionic strength, on the specificity for the acidic phospholipid cardiolipin and on the pH dependence is considered to indicate that cytochrome *c* is bound electrostatically to the polar-head groups of the phospholipid molecules. If one accepts a bimolecular lipid leaflet (Gorter & Grendel, 1925) as the structure of the individual lipid membranes, then cytochrome *c* would be localized on the surface polar groups, rather than in the hydrophobic interior of the bilayer. In this model, an increase in width corresponding either to the 31-A diameter (Dickerson, Kopka, Bordens, Varnum, Weinzierl & Margoliash, 1967) or to some multiple of the diameter of the roughly spherical cytochrome *c* molecule might be expected. The increase in X-ray diffraction spacing in the presence of cytochrome *c* (Papahadjopoulos & Miller, 1967; Shipley *et al.*, 1969*b*; Gulick-Krzywicki *et al.*, 1969) and the increase in lamella width measured in the electron microscope studies presented in this paper agree with this anticipated finding.

The actual values found in this work for center-to-center distances of the repeating units were 45 and 88 A in the absence and presence of cytochrome *c*, respectively. This compares with values from X-ray diffraction studies of 87- and 116-A spacing in the presence of cytochrome *c*. Since the preparation of the electron micrographs undoubtedly involves some dehydration, values based on electron microscope studies can be expected to be minimal. The center to-center measurements are nonetheless in good agreement with the values from X-ray diffraction on wet preparations in the presence of cytochrome *c*. The center-to-center distance of 45 A in the absence of cytochrome *c* is in good agreement with the values of 40 A obtained from electron micrographs of osmium tetroxide-stained thin sections of a phospholipid brain extract (Stoeckenius *et al.*, 1960). According to Stoeckenius *et al.* (1960), the optically dense areas represent the polar head groups of the phospholipids. In these liquid crystal structures, one dense line probably corresponds to the closely apposed polar head groups from the phospholipids of two neighboring bilayers, the white line representing the hydrophobic interior of the bilayer. Following this interpretation, the presence of cytochrome *c* should increase only the electron-dense line if it is only adsorbed onto the polar surface. A comparison of Figs. 5 and 7 would indicate that cytochrome *c* does indeed increase the width of the

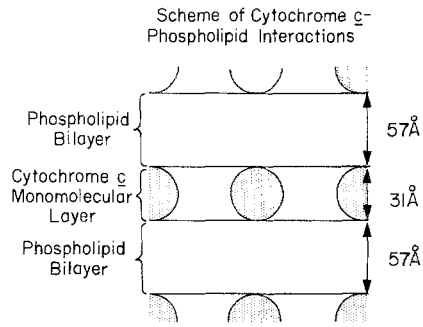

Fig. 9. Scheme of postulated cytochrome *c*-phospholipid interactions. Shaded circles represent the approximately spherical cytochrome *c* molecules; open areas between lines represent bimolecular phospholipid membranes, with the polar-head groups on the surfaces

dense line. For reasons outlined by others (Stoeckenius *et al.*, 1960), quantitative measurements of the width of these lines is unreliable. The increase in width shown in Fig. 7 appears to be uniformly distributed. This is different from the findings of Stoeckenius *et al.* (1960) for the addition of globin to their phospholipid dispersions where an increase in width of only the outermost dense line was seen. In their case, however, the globin may have been added after the myelin figures had formed.

Consideration of the amount of cytochrome *c* that is bound to the vesicles and the space it would occupy if distributed evenly on the surface of each bilayer provides alternative criteria for the change in dimensions to be expected. The maximum amount of cytochrome *c* bound that is obtained (*see* Fig. 1A) is 0.170 μmole cytochrome *c*/10.4 μmoles lecithin *plus* 1.45 μmoles cardiolipin. For these proportions, and taking 55 A^2 per molecule of lecithin as an approximate area in the bimolecular membranes from the collapse pressure found in monolayers (Papahadjopoulos, 1969), and 110 A^2 for cardiolipin (Shah & Schulman, 1965), the area of phospholipid will be 572 A^2 *plus* 160 $A^2 = 732$ A^2. For the spherical cytochrome *c* molecule of 31-A diameter, the proportional cross-sectional area will be 128 A^2. This would permit cytochrome *c* to be arranged as a monomolecular layer between two phospholipid bilayers, occupying 36% of the total membrane area. It is also approximately equivalent to the amount of cardiolipin present. This possible arrangement is shown in Fig. 9. Thus, this is consistent with cytochrome *c* increasing the width of the membrane by the diameter of one cytochrome *c* molecule, it if is adsorbed on the surface polar groups of the phospholipids.

The figure of 57 A for the thickness of the phospholipid bilayer in Fig. 9 is obtained from the subtraction of the 31-A diameter of cytochrome

c from the 88-A value obtained for center-to-center distance measurements for two neighboring light or dense lines in the presence of cytochrome *c*. This is greater than the actual value obtained for such measurements in the absence of cytochrome *c*, but this value may be lower due to shrinkage and dehydration during the preparation of the samples for electron microscopy, the effects of which may be minimized when cytochrome *c* is present. For a double layer of cytochrome *c* between each bilayer, the center-to-center distance of 88 A would leave 26 A for the width of the phospholipid bilayer. Shipley *et al.* (1969*b*) and Gulik-Krzwicki *et al.* (1969), using X-ray diffraction studies, have indicated the existence of both types of arrangement, a single and double molecular layer of cytochrome *c* between each phospholipid bilayer, for their preparations. Both structures may have existed in our preparations and become altered and condensed during the dehydration and embedding procedures. It is clear that the electron microscope measurements do not permit any definitive distinction between these various possible models. They do, however, show that an increase in membrane width does occur and seems to affect mainly the electron-dense polar-head-group area of the phospholipid bilayer. These results together with the more precise values obtainable from X-ray diffraction measurements support the concept that cytochrome *c* is adsorbing on the surface of the membrane.

The question of the biological relevance of this model system, as far as can be determined from the examination of selected biochemical parameters, is pursued in the following paper (Kimelberg & Lee, 1970).

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References

Bangham, A. D. 1968. Membrane models with phospholipids. *In*: Progress in Biophysics and Molecular Biology. J. A. V. Butler and D. Noble, editors. p. 18. Pergamon Press, New York.

— Standish, M. M., Watkins, J. C. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **13**:238.

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466.

Das, M. L., Crane, F. L. 1964. Proteolipids. I. The formation of phospholipid-cytochrome *c* complexes. *Biochemistry* **3**:696.

— Haak, E. D., Crane, F. L. 1965. Proteolipids. IV. Formation of complexes between cytochrome *c* and purified phospholipids. *Biochemistry* **4**:859.

Dickerson, R. E., Kopka, M. L., Borders, C. I., Varnum, J., Weinzierl, J. E., Margoliash, E. 1967. A centrosymmetric projection at 4 Å of horse heart oxidized cytochrome *c*. *J. Mol. Biol.* **29**:77.

Gorter, E., Grendel, F. 1925. On bimolecular layers of lipoids on the chromocytes of the blood. *J. Exp. Med.* **41**:439.

Green, D. E., Fleischer, S. 1963. The role of lipids in mitochondrial electron transfer and oxidative phosphorylation. *Biochim. Biophys. Acta* **70**:554.

Gulik-Krzywicki, T., Schechter, E., Luzzati, V., Faure, M. 1969. Interactions of proteins and lipids: Structure and polymorphism of protein-lipid-water phases. *Nature* **223**:1116.

Jacobs, E. E., Sanadi, D. R. 1960. The reversible removal of cytochrome *c* from mitochondria. *J. Biol. Chem.* **235**:531.

Kimelberg, H. K., Lee, C. P. 1969. Binding and electron transfer to cytochrome *c* in artificial phospholipid membranes. *Biochem. Biophys. Res. Commun.* **34**:784.

— — 1970. Interactions of cytochrome *c* with phospholipid membranes. II. Reactivity of cytochrome *c* bound to phospholipid liquid crystals. *J. Membrane Biol.* **2**:252.

Lee, C. P., Carlson, K. 1968. Binding of cytochrome *c* to fragmented mitochondrial membranes. *Fed. Proc.* **27**:828.

— Ernster, L. 1966. Energy-linked pyridine nucleotide transhydrogenase: Its characteristics and its use as a tool for the study of oxidative phosphorylation. *In*: Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater, editors. BBA Library. **7**:218.

Lenaz, G., MacLennan, D. H. 1966. Studies on the mechanism of oxidative phosphorylation. X. The effect of cytochrome *c* on energy-linked processes in submitochondrial particles. *J. Biol. Chem.* **241**:5260.

Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.

Machinist, J. M., Das, M. L., Crane, F. L., Jacobs, E. E. 1961. A negatively charged pore in the mitochondrial membrane as a site for cytochrome *c* function. *Biochem. Biophys. Res. Commun.* **6**:475.

Margoliash, E., Frohwirt, N. 1959. Spectrum of horse heart cytochrome *c*. *Biochem. J.* **71**:570.

Nicholls, P., Mochan, E., Kimelberg, H. K. 1969. Complex formation by cytochrome *c*: A clue to the structure and polarity of the inner mitochondrial membrane. *Fed. Europ. Biochem. Socs. Letters* **3**:242.

Papahadjopoulos, D. 1968. Surface properties of acidic phospholipids: Interaction of monolayers and hydrated liquid crystals with uni- and bivalent metal ions. *Biochim. Biophys. Acta* **163**:240.

— Miller, N. 1967. Phospholipid model membranes· I. Structural characteristics of hydrated liquid crystals. *Biochim. Biophys. Acta* **135**:624.

Parsons, D. F., Williams, G., Thompson, W., Wilson, D., Chance, B. 1967. Improvements in the procedure for purification of mitochondrial outer and inner membrane. Comparison of the outer membrane with smooth endoplasmic reticulum. *In*: Mitochondrial Structure and Function. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, editors. p. 29. Adriatica Editrice, Bari.

Reich, M., Wainio, W. W. 1961. A cytochrome *c*-phospholipid complex. *J. Biol. Chem.* **236**:3058.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.

Schneider, W. C., Claude, A., Hogeboom, G. H. 1948. The distribution of cytochrome *c* and succinoxidase activity in rat liver fractions. *J. Biol. Chem.* **172**:451.

Shah, D. S., Schulman, J. H. 1965. Binding of metal ions to monolayers of lecithins, plasmolagen, cardiolipin, and dicetyl phosphate. *J. Lipid Res.* **6**:341.

Shipley, G. G., Leslie, R. B., Chapman, D. 1969*a*. Small-angle X-ray scattering studies of cytochrome *c*-phospholipid complexes. *Biochim. Biophys. Acta* **173**:1.

— — — 1969*b*. X-ray diffraction study of the interaction of phospholipids with cytochrome *c* in the aqueous phase. *Nature* **222**:561.

Stoeckenius, W., Schulman, J. H., Prince, L. M. 1960. The structure of myelin figures and microemulsions as observed with the electron microscope. *Kolloidzschr.* **169**:170.

Theorell, W., Åkesson, A. 1941. Studies on cytochrome *c*. II. The optical properties of pure cytochrome *c* and some of its derivatives. *J. Amer. Chem. Soc.* **63**:1804.

Tsou, C. L. 1952. Exogenous and endogenous cytochrome *c*. *Biochem. J.* **50**:493.