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A Study of the Ultrastructural Organization of Cytochrome *c*-Phospholipid Membranes as Revealed by Various Experimental Treatments

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Summary. Two different types of cytochrome *c*-phospholipid model membranes have been investigated by electron-microscopy. The preservation of the lamellar structure of the cytochrome *c* plus phospholipid-water system after extraction of 88% phospholipids with aqueous acetone followed by only aldehyde fixation is revealed.

Pronase digestion of this type of model membrane leads to removal of 80% of the protein and loss of ordered structures as revealed by aldehyde post-fixation.

It is shown that the hydrated isooctane-soluble cytochrome *c*-phospholipid complex retained the lamellar structures after extraction of 75% phospholipids followed by separate aldehyde and osmium tetroxide fixations. Pronase treatment of this type of model membrane leads to removal of 25 to 30% of the protein. After aldehyde fixation of these pronase-treated samples the triple-layered structures are preserved.

The structural integrity of both types of model membranes after organic extractions with aldehyde pre- or post-fixation is thought to be the result of cross-linkage of the protein by aldehyde polymers on both surfaces and through lipid bilayers. Possible localization of cytochrome *c* in investigated model membranes is discussed.

Different physical methods give direct experimental evidence that lipid components of cell membranes are organized as a bimolecular layer (Hendler, 1971; Wilkins, Blaurock & Engelman, 1971). However, many problems dealing with the structural associations between lipid and protein molecules in biological membranes remain unclear. Some information in this respect may be obtained from ultrastructural investigations of model membranes with which some features of the cell membranes can be reproduced (Malhotra, 1970).

In recent years, many papers considering some aspects of the interaction between cytochrome *c* and phospholipids have been published (Gulik-Krzywicki, Shechter, Luzzati & Faure, 1969; Quinn & Dawson, 1969, 1970; Kimelberg & Lee, 1970; Kimelberg, Lee, Claude & Mrena, 1970; Stoeckenius, 1970; Kimelberg & Papahadjopoulos, 1971*a*, *b*; Steinemann & Läuger,

1971; Blaurock, 1972; Lenaz, Pasquali, Bertoli, Sechi, Parenti-Castelli & Masotti, 1972).

Cytochrome *c*, a globular protein, is known to carry eight positive charges and to interact with negatively charged and neutral phospholipids in water with a molar protein/lipid ratio of 1:70 to 1:300 at neutral pH (Hart, Leslie, Davis & Lawrence, 1969). It is also known (Das & Crane, 1964; Sun & Crane, 1969) that the cytochrome *c*-phospholipid complex may be extracted from water subphase by nonpolar solvents. The molar protein/lipid ratio of such proteolipids is 1:20 to 1:30. From ORD (Ulmer, Valley, Gorchein & Neuberger, 1965) and spin label studies (Barrat, Green & Chapman, 1968) it is known that the conformational states of cytochrome *c* molecules are similar in water solution and in the isooctane-soluble cytochrome *c*-phospholipid complex. When this proteolipid complex is transferred into water, the hydrated proteolipid complex of an ordered structure is formed, which may have lamellar (Sun & Crane, 1969) and hexagonal (Borovjagin & Moshkov, 1970) phases. The protein/lipid molar ratio in the isooctane-soluble cytochrome *c*-phospholipid complex is more constant than in the systems where cytochrome *c* is added to lipid-water dispersions (Shipley, Leslie & Chapman, 1969; Sun & Crane, 1969). This fact may be regarded as indirect evidence of more specific interaction between the protein and lipids in former complexes. Moreover, it has been demonstrated (Sun & Crane, 1969) that cytochrome *c* in the hydrated proteolipid complex has a high electron transfer activity comparable with that observed in mitochondrial membranes. However, nothing is known about the structural interaction of cytochrome *c* with phospholipids in such a complex.

Information from comparative ultrastructural studies of the hydrated proteolipid complex and cytochrome *c* plus phospholipid-water system under the experimental treatments commonly used for investigating cell membranes seems to be useful for understanding the structural principles underlying these model membranes.

In this paper we present an investigation of the fine structure of these two types of model membranes after organic extractions and enzymatic hydrolysis. Besides, in this work we should like to emphasize the significance of aldehyde fixations for the ultrastructural integrity of lipoprotein artificial membranes.

Materials and Methods

Chemicals

Horse heart cytochrome *c*, 25% glutaraldehyde and dried *p*-formaldehyde were obtained from Schuchardt (München); 3-mercaptopropanol was purchased from Koch-

Light (England); crystalline pronase from *Streptomyces grisens* was obtained from Nagase (Japan); measured activity was 150,000 PUK/g. All other chemicals were obtained commercially and were analytical grade or of the purest quality.

Preparations

Mitochondrial lipids were extracted with chloroform-methanol, essentially according to Rouser and Fleischer (1967), from freshly isolated rat liver or pig heart mitochondria. These lipids, being completely soluble in aqueous acetone, required no further purification in most cases.

Ox brain phospholipids were extracted from ox brain white matter according to Mueller, Rudin, Tien and Westcott (1964). The lipid extracts as checked by thin-layer chromatography (t.l.c.) on Silica gel G (Merck), contained all specific classes of phospholipids and were kept at -10 °C under argon as chloroform-methanol solutions.

Acetone reextraction of ox brain phospholipids. The initial chloroform-methanol lipid solutions were dried under vacuum and every solid residue was treated with acetone (Fleischer, Fleischer & Stoeckenius, 1967) step by step: 4% water in acetone, 10% water in acetone and 10% water in acetone plus 13.5 μliters of ammonia hydrochloride (25% v/v) per 100 ml of the mixture. Each step of the treatment was performed at 20 °C for 1 hr. All acetone solutions were summarized, filtered and concentrated to small volumes in a vacuum evaporator and were kept (overnight) under argon at 10 °C.

To estimate the average molecular weight of liposome phospholipids, the dry weight determination and analysis for phosphate were made with the same samples.

The isooctane-soluble cytochrome c-phospholipid complex was prepared from mitochondrial phospholipids and acetone-soluble fraction of brain phospholipids. This procedure was carried out essentially as described elsewhere (Das & Crane, 1964). The proteolipid in isooctane containing all origin classes of phospholipids, as revealed by t.l.c., was stored before use at -10 °C under argon.

Liposomes were prepared as follows: a small amount of initial mitochondrial phospholipid solution was evaporated in a glass tube to dryness; the buffer solution (0.05 M Tris-HCl, 0.015 M NaCl and 1 mM 3-mercaptopropanol, pH 7.0 to 7.4) was added and the tube was mechanically shaken. In the case of brain phospholipids, the liposomes were prepared by dilution of stock phospholipid solutions with the same buffer. Both types of liposomes will be referred to as "big" liposomes. "Small" liposomes were prepared by ultrasonic microdispersion of "big" liposomes in a USDM-I apparatus at 15 kHz, 100 watts, over the total time of 30 min (ice bath, continuous gassing of the solutions with argon; the temperature did not exceed $+10$ °C).

The artificial phospholipid membranes containing the cytochrome c-lipoprotein model (Lpm) membranes were prepared by mixing (vigorous stirring): 2.5 ml of 2% appropriate "big" or "small" liposome solution with 0.5 ml absolute ethanol and 0.5 ml cytochrome *c* (5 mg/ml) in 0.05 M Tris-HCl buffer (pH 7.4). Ethanol was added to accelerate the complex formation (Das & Crane, 1964) and washed off afterwards. The resulting mixtures were diluted with the same buffer to make a final volume of 30 ml and were centrifuged two times. The pellets were then washed (with recentrifugation after each washing) with 30 ml 0.15 M NaCl solution to remove the excess of cytochrome *c* from Lpm membranes as described by Kimelberg *et al.* (1970). The final pellets were used for organic extraction experiments. In other cases these pellets were diluted with the buffer solution.

The proteolipid model (Plm) membranes were prepared from the isooctane-soluble cytochrome *c*-phospholipid complex as described elsewhere (Sun & Crane, 1969). Isooctane was evaporated to dryness in a vacuum. The same buffer solution (as in the case of liposome preparations) was added to give a final concentration of approximately 2 mg dry weight in 100 ml. A rather hydrophobic "solid" complex was scraped from the bottom of the tube into buffer solution and was mechanically (large crystals) or ultrasonically (small vesicles) dispersed as described in the section dealing with the preparation of liposomes. In some cases incubation at 37 °C (1 to 2 hr) was performed to accelerate the swelling of large crystals.

Experimental Procedures

The accessibility of multibilayer phospholipids to cytochrome c was tested as follows: the buffer solution with "big" and "small" liposomes was mixed [by gently stirring it once with a cytochrome *c* solution and allowing it to stand for 3 hr at room temperature (sometimes at 37 °C)]. The amount of the protein was twice as high as that required for interaction with the bulk of phospholipids to proceed. "Big" and "small" liposomes with no cytochrome *c* added served as control samples. After incubation, each sample was divided in half. Glutaraldehyde was added to one portion (the final concentration of aldehyde was 2.5%) and osmium tetroxide (the final concentration was 1%) to the other. The samples were fixed for 1 to 24 hr, centrifuged, and the sediments were subjected to an ultrastructural analysis (*see* section on "Electron-microscopy").

Organic solvent extraction experiments. Freshly prepared "big" liposomes containing cytochrome *c* (the protein/phospholipid molar ratio was 1:60) and large crystals of Plm membranes (protein/phospholipid molar ratio was 1:30) were treated with acetone: (a) essentially according to the procedures described in the section "Acetone reextraction of ox brain phospholipids"; in some cases after the third step treatment (10% water in acetone plus 13.5 μliters NH_4OH) washing with 10% water in acetone with 10 N HCl (100:0.33 v/v) for 1 hr was used; (*b*) exactly as in the acetone dehydration procedure (*see* "Electron-microscopy").

The Lmp and Plm membranes pre-fixed with aldehydes (*see* "Electron-microscopy") were treated essentially according to Napolitano, LeBaron and Scaletti (1967); acetone dehydration, extraction with chloroform-methanol (2:1 v/v) followed by chloroform-methanol-HCl (200:100:1 v/v/v) treatment. Organic solvents and solid residues were stored for further chemical and morphological analyses.

Pronase hydrolysis experiments. Pronase at a final concentration of 50, 125 or 250 μg/mg protein of preparation was added to ultrasonic solubilized Plm and Lpm membrane suspensions in 0.05 M Tris-HCl buffer, containing 3 mM 3-mercaptopropanol. The incubations were carried out in centrifuge tubes at 37 °C for 1 to 20 hr. To each tube one drop of toluene was added. The following experiments served as control: (a) incubation of the initial model membranes in the same conditions without pronase; (b) incubation of "small" liposomes in the same conditions with pronase (250 μg/ml suspension). To stop the action of pronase the samples were diluted after incubation with a cold buffer solution and centrifuged for 1 hr at 160,000 × g. The supernatants and sediments were collected and stored for further protein and ultrastructural analyses.

The action of pronase on pure cytochrome *c* was tested by incubating a cytochrome *c*-pronase solution in the above mentioned conditions and dry weight determinations of the sediments (at centrifugation 50,000 × g for 30 min) after 10% trichloroacetic acid precipitation (Broam, Harmsen, Walters & Van Os, 1971).

Chemical analyses. A quantitative determination of phospholipids was performed as described by Chen, Toribara and Warner (1956). The qualitative control analysis, using the t.l.c. technique according to Gigg and Payne (1969), is published elsewhere (Borovjagin, Medvedev & Moshkov, 1972). The content of cytochrome *c* in the samples (resulting sediments and summarized supernatants) was measured according to Lowry, Rosebrough, Farr and Randall (1951) or by the reduced-minus oxidized spectra with a split-beam spectrophotometer (SP-800) as described by Kimelberg *et al.* (1970). In the proteolipid complex, cytochrome *c* was determined from the absorbancy in isooctane at 407 nm.

Electron-microscopy. The ultrastructural and chemical analyses were carried out in every experiment. All suspensions or sediments after the last experimental treatment were fixed at 18 to 20 °C (sometimes at 37 °C) separately: (a) 1% or 2.5% glutaraldehyde for 1 to 24 hr; (b) 1% formaldehyde [prepared from *p*-formaldehyde according to Burgos, Vitale-Calpe and Téllez de Iñon (1967)] for 1 to 24 hr; (c) 1% osmium tetroxide for 1 to 2 hr. All fixatives were buffered by 0.05 M Tris-HCl solution (pH 7.0 to 7.4) and made isotonic by adding NaCl. In some experiments a part of the material was not fixed at all. In all cases the fixed or unfixed samples were recentrifuged (8,000 rpm for 15 min). Dehydration of the sediments was accomplished in 30, 50, 75, 85, and 95% acetone and four changes of anhydrous acetone, the total time being 120 min.

After dehydration the pellets were infiltrated with Epon-812 and polymerized at 37 and 60 °C. After aldehyde pre-fixation followed by chloroform-methanol delipidization, the sediments were rinsed in absolute ethanol, then placed in anhydrous acetone. Further steps were similar to the procedures described above. Thin sections were cut on an Ultratome-III (LKB) and stained with saturated water solution of uranyl acetate for 45 or 120 min and lead citrate for 15 or 30 min. The sections were examined in the JEM-7A electron-microscope at 80 kV with 30 μ objective aperture. The photographs were made at a 15,000 to 80,000 plate magnification.

Results

The Accessibility of Multibilayer Phospholipids to Cytochrome c

Investigation of the cross-sections of "big" and "small" liposome suspensions incubated with cytochrome *c* (for 3 hr) followed by osmium tetroxide fixation has revealed two types of the lamellar structures with repeat distance of 75 to 78 Å (Lpm membranes) and 45 to 48 Å (pure lipid phase), as seen in Fig. 1*a*. After aldehyde fixations of the same samples the Lpm membranes with a repeat distance of 85 Å were revealed (Fig. 1*b*). In this case the lamellar structures (3 to 5 lamellae) were localized from the outside surface of the liposomes. The hydrophilic (intensively stained) regions of this multilayered structure were often divided into halves by light gaps (Fig. 1*a*, *a'* and *b*, arrows). After incubation of the samples at 37 °C the gaps become pronounced (Fig. 1*a'*). The thickness of a single Lpm membrane in the osmium-fixed suspensions was found to be 78 to 80 Å and in those fixed with aldehyde, 90 to 95 Å (Fig. 1*c*).

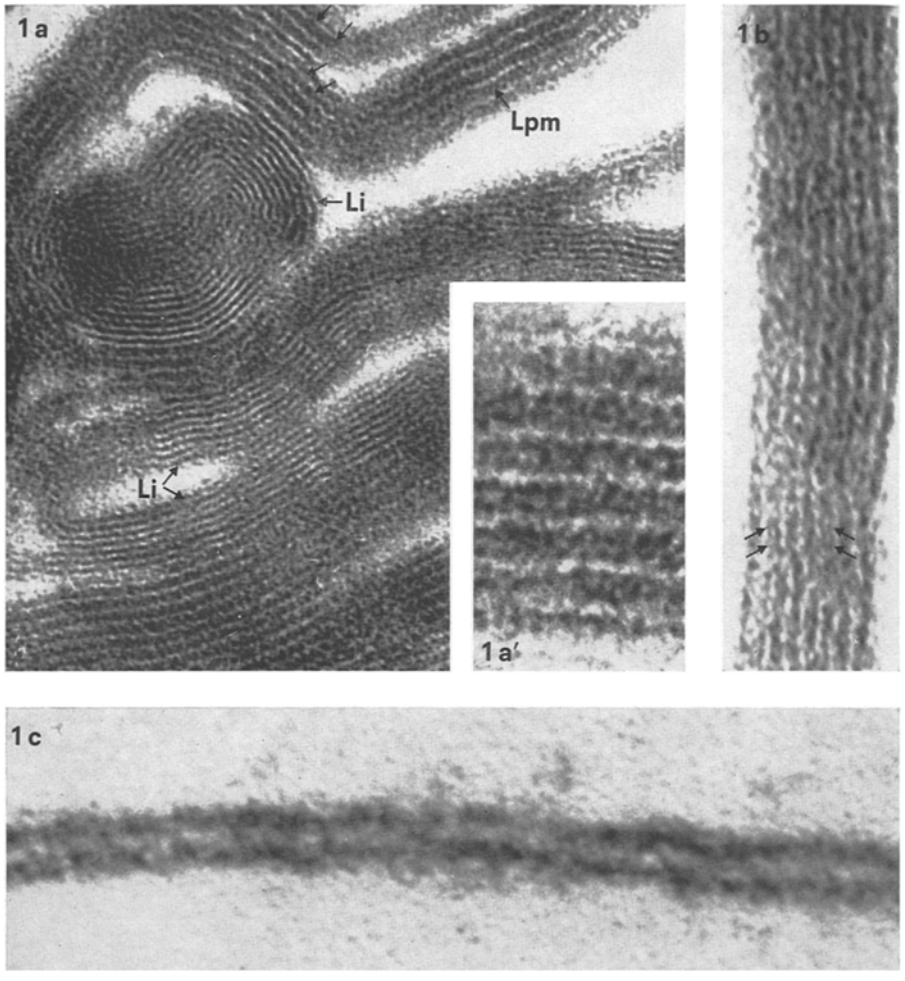

Fig. 1. (*a*) The lamellar structure of a "big" liposome incubated in the presence of cytochrome *c* for 3 hr. Osmium tetroxide fixation. The lipoprotein model membrane (Lpm) and lipid bilayer (Li) structures are seen. Note interlamellar gaps (arrows); ×295,000. (*a'*) The samples of Lpm incubated at 37 °C for 2 hr. The interlamellar gaps are clearly seen; ×950,000. (*b*) The same as in Fig. 1*a*, glutaraldehyde fixation. Note interlamellar gaps (arrows); ×440,000. (*c*) The lipoprotein model membrane, formaldehyde fixation; ×900,000

The Structure of the Plm Membranes

Large crystals. When unordered isooctane-soluble cytochrome *c* phospholipid complex was transferred into the water phase, various stages of the Plm membrane formation have been observed on the cross-sections of the samples after both aldehyde and osmium tetroxide fixations (Fig. 2*a*,

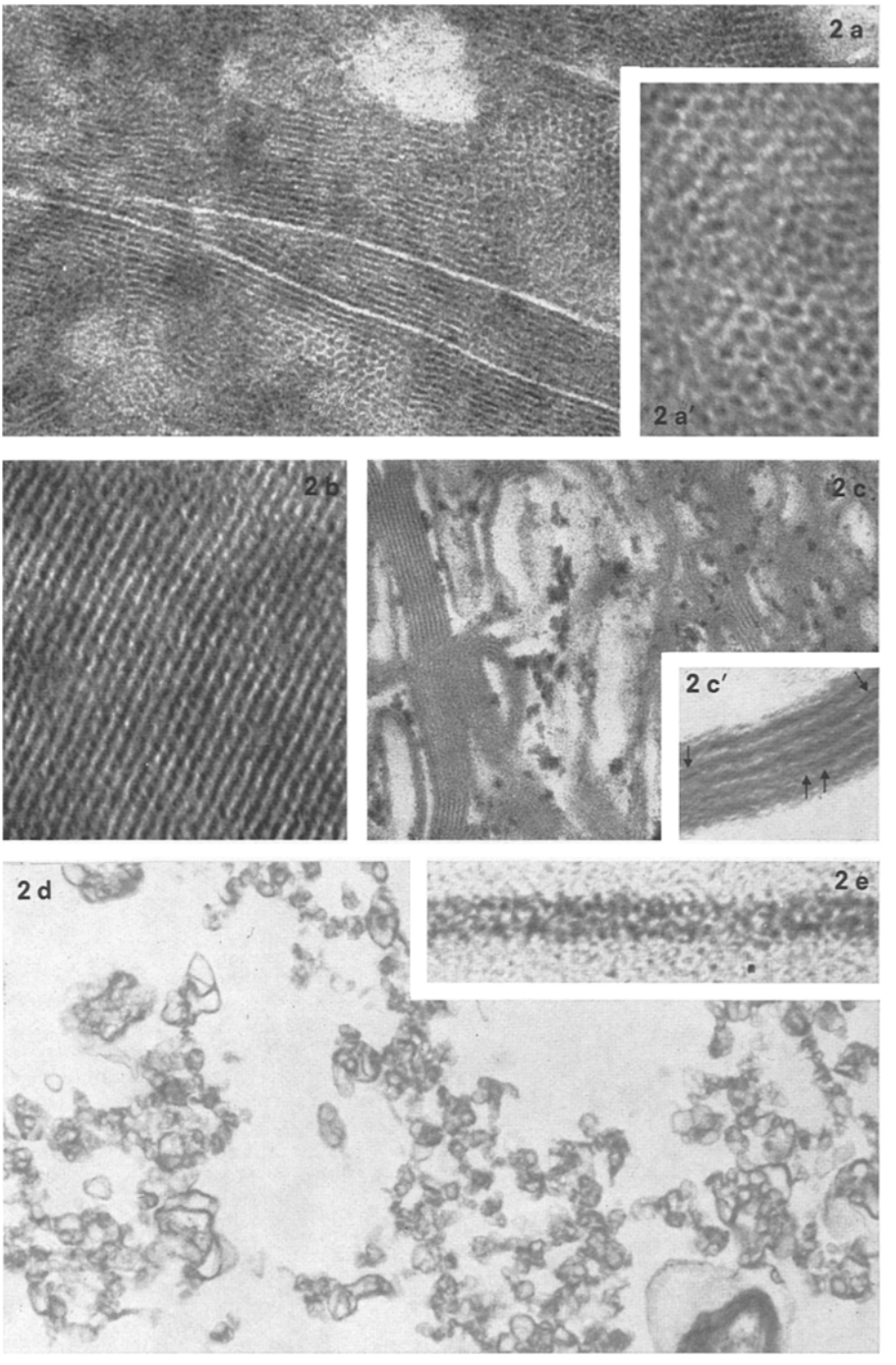

Fig. 2. (*a*, *a'*) The lamellar and hexagonal structures of the proteolipid model membrane at the initial stage of swelling: (*a*) osmium tetroxide fixation; ×200,000; (*a'*) hexagonal structures, glutaraldehyde fixation; ×430,000. (*b*) The lamellar structures of a large crystal (proteolipid model membranes). Osmium tetroxide fixation; ×390,000. (*c*, *c'*) The lamellar structures of proteolipid model membranes after incubation at 37 °C for 3 hr. Note interlamellar gaps (arrows); osmium fixation; (*c*) ×110,000; (*c'*) ×375,000. (*d*) A cross-section of sonicated proteolipid model membranes. Osmium fixation; ×60,000. (*e*) The proteolipid model membrane of a single wall vesicle incubated at 37 °C for 20 hr (a control sample from a pronase treatment experiment). Glutaraldehyde fixation; ×700,000

a', *b* and *c*). A hexagonal (tubular) structure with a center distance of 90 to 95 Å (Fig. 2*a* and *a'*) and a lamellar phase with a repeat distance of about 75 to 77 Å (Fig. 2*b*) were formed at the initial stages of swelling. The samples which had been in water for a longer time (or at 37 °C) exhibit only compact (Fig. 2*b*) or loose (Fig. 2*c*) lamellar structures with interlamellar (hydrophilic region) gaps (Fig. 2*c*; 2*c'*, arrows).

Small vesicles. The ultrastructure of sonicated large crystal suspensions is demonstrated in Fig. 2*d*. Small vesicles were bound to one or several layers having the dimension of single Plm membranes, about 80 Å (Fig. 2*e*).

Organic Extraction Experiments

Acetone extractions. Table 1 summarizes the effect of acetone extraction on the phospholipid content of Lpm and Plm membranes.

"A" includes one step of acetone treatment; "B" includes the "A" step followed by the "B" step treatment; the "C" step includes the "B" step followed by the "C" step treatment; the "D" includes all four steps of acetone treatment.

An ultrastructural study shows that after the "A" and "B" step treatments (*see* Table 1) followed by osmium tetroxide or aldehyde fixation, the lamellar structures in both types of model membranes are well preserved (Fig. 3*a*). After the "C" step treatment of the samples followed by aldehyde

Table 1. Phospholipid content of model membranes

Treatment 20 °C	Type of model membrane [a]			
	Lpm membranes		Plm membranes	
	(μg P in samples)	(%)	(μg P in samples)	(%)
None (control)	69.5	100	41.0	100
4% water in acetone ("A" step)	17.5	25.2	15.6	38.0
10% water in acetone ("B" step)	12.3	17.7	12.4	30.2
10% water in acetone plus NH_4OH ("C" step)	8.3	11.9	10.2	24.8
10% water in acetone plus HCl ("D" step)	6.2	8.7	4.0	10.4

[a] Figures are averages of the results obtained from 6 samples (only brain phospholipids were used).

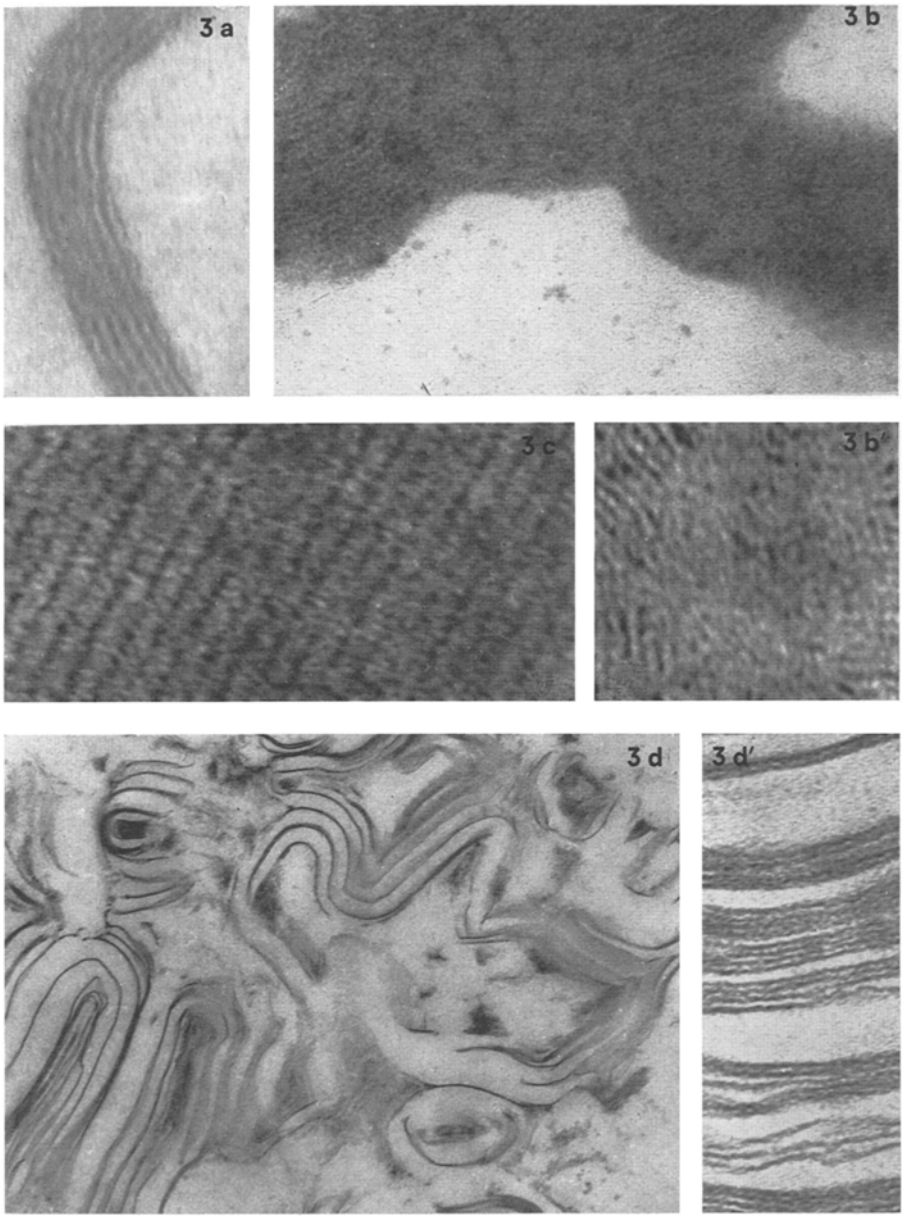

Fig. 3. (*a*) The lamellar structure of lipoprotein model membranes after "A" and "B" steps (*see* Table 1) of acetone treatment (82% phospholipids removed) followed by osmium tetroxide fixation; ×290,000. (*b*, *b'*) The lamellar proteolipid model membranes after the "A", "B" and "C" steps of acetone treatment (75% phospholipids removed) followed by glutaraldehyde fixation (*b*, ×235,000) or osmium tetroxide fixation (*b'*, ×360,000). (*c*) The lamellar lipoprotein model membranes after the "A", "B" and "C" steps of acetone treatment (88% phospholipids removed) followed by glutaraldehyde fixation; ×570,000. (*d*, *d'*) A cross-section of the lipoprotein model membranes dehydrated with acetone and Epon embedded without fixation; (*d*, ×60,000; *d'*, ×240,000)

fixation the layered structure can be revealed in both types of model membranes (Fig. 3*b* and *c*). If we used osmium tetroxide fixation after this treatment step the lamellar patterns could be seen only in the Plm membranes (Fig. 3*b'*) and were disrupted in the Lpm membranes. After the "D" step treatment both types of model membranes being transferred into fixatives were completely dissolved.

In some experiments freshly prepared samples of both types of model membranes were dehydrated in a graded series of acetone (*see* "Materials and Methods," "Electron-microscopy") and then were embedded in Epon without fixation. On the cross-sections of Lpm membranes rather good preservation of lamellae (with great variations in the size of single lamella) was observed (Fig. 3*d* and *d'*), but in the Plm membranes no ordered structures were detected.

The cross-section parameter of single model membranes and the repeat distance in multilayered structures in all the samples after all steps of acetone extraction followed by aldehyde fixation were slightly less then in the unextracted control samples. Sometimes a great degree of size variation was observed. In all the cases when aldehyde fixations preceded any kind of acetone treatments, on the cross-sections of the embedded samples good multilamellar (Fig. 1*b*), hexagonal (Fig. 2*a'*) and single triple-layered fine structures (Fig. 1*c*) were observed. It is necessary to point out that the degree of integrity of the ordered structures correlated with the conditions of fixation: the longer the time and/or the higher the temperature the better preserved the fine structures were.

Chloroform-methanol extractions. Treatment of the unfixed Plm and Lpm membranes with chloroform-methanol mixture completely dissolved them. Therefore, these procedures were performed only with aldehyde pre-fixed material. In these experiments the quantitative analyses for phosphate were omitted. The qualitative phospholipid determination by t.l.c. revealed that all cephalines remained in the samples after aldehyde fixation and organic extractions (Borovjagin *et al.*, 1972). An electron-microscopic examination of this material showed well-preserved lamellar structures (Fig. 4*a*) in both types of investigated samples, but great variation in the size of cross-section of single lamella was frequently seen (Fig. 4*a'*).

We should emphasize that, generally, the preservation of membranous structures in this case strongly depended not only upon the time and temperature of fixation, but also, to a great extent, on the nature of aldehyde: glutaraldehyde gave much better results than formaldehyde.

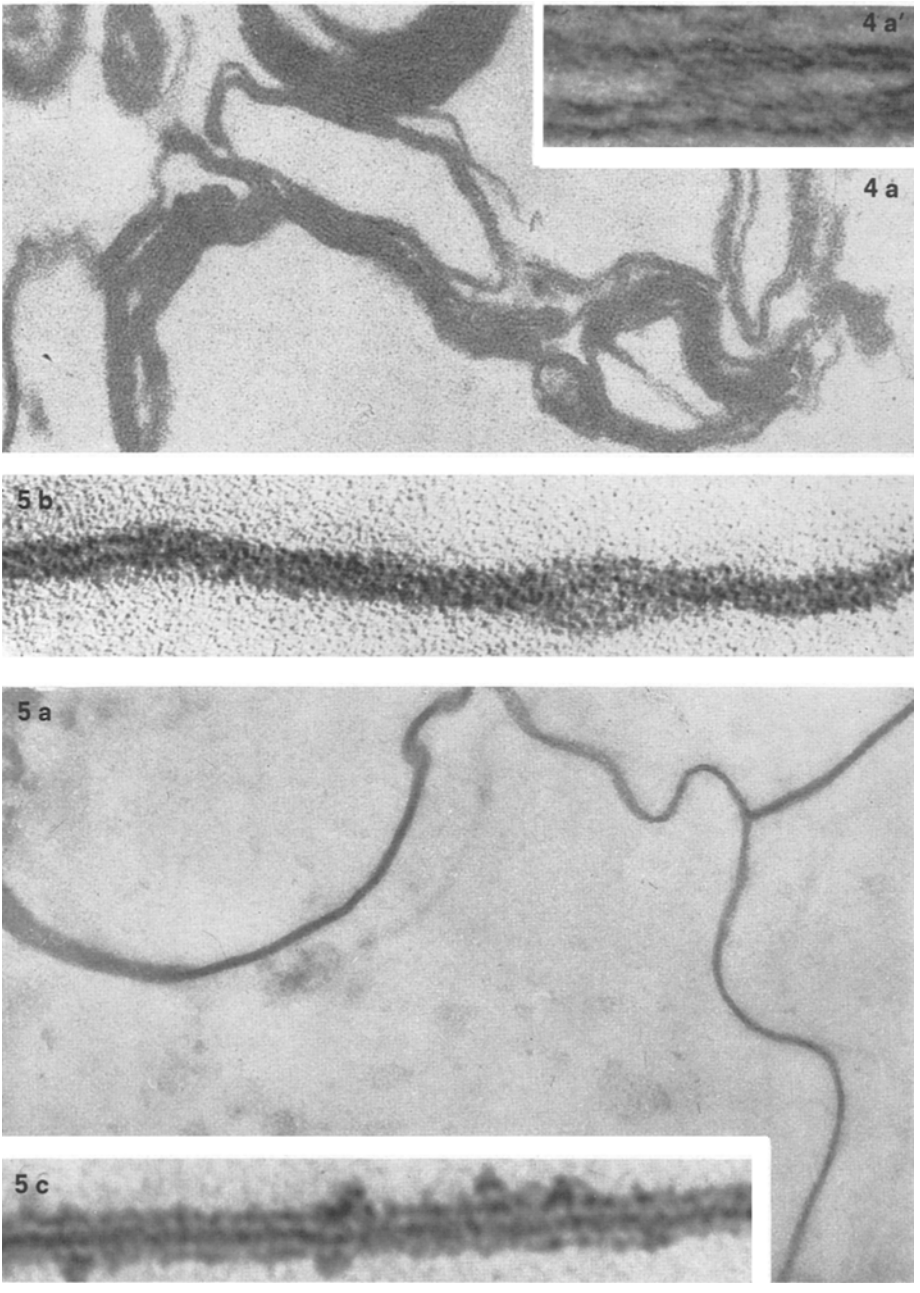

Fig. 4. (*a*, *a'*) Glutaraldehyde-fixed chloroform-methanol-HCl extracted lipoprotein model membranes. (*a*, × 100,000; *a'*, × 500,000)

Fig. 5. (*a*, *b*) The proteolipid model membranes after incubation with pronase (250 μg/mg Pr. for 20 hr at 37 °C) followed by glutaraldehyde fixation. (*a*, × 75,000; *b*, × 700,000). (*c*) The same preparation as in (*a*); osmium tetroxide fixation; × 560,000

Pronase-Treated Model Membranes

After incubation of pure cytochrome *c* solutions with pronase (250 μg/mg protein) for 2 hr, complete digestion of protein took place. In the samples of "small" liposomes incubated with pronase (250 μg/ml, for 10 hr or more) followed by aldehyde fixation and acetone dehydration no ordered or vesicular structures were seen. In the experiments with pronase-treated Lpm and Plm membranes, the effect of hydrolysis was found to be maximal with an enzyme concentration of 250 μg/mg protein and directly depended on the time of incubation. The treatment of suspensions with the enzyme (250 μg/mg protein) for 20 hr resulted in about 80% of the protein being digested in the Lpm membrane samples and about 25 to 30% of the protein being hydrolyzed in the Plm membranes. When the Lpm membrane suspensions after incubation in such conditions were fixed with aldehyde, only amorphous material could be observed, just as osmium tetroxide post-fixation of these samples revealed "foamy" bilayer lipid structures. An electron-microscopic investigation of Plm membranes after an identical incubation with pronase, aldehyde post-fixation and acetone dehydration revealed aggregated vesicles (Fig. 5*a*) bound to one (Fig. 5*b*), two (Fig. 5*c*) or several triple-layered structures to be present in these preparations. The dimensions of these model membranes were almost indistinguishable from those of the control samples (Fig. 2*e*).

Addendum: No essential differences in the structural properties of the model membranes prepared from either mitochondrial or brain phospholipids were revealed.

Discussion

A comparative analysis of the cross-sections of the "big" and "small" liposome suspensions after 3 hr of incubation with cytochrome *c* followed by separate aldehydes and osmium tetroxide fixations leads to the conclusion that this protein displays a low accessibility to phospholipid multilayers.

There are some possibilities that cytochrome *c* molecules are localized between lipid bilayers. Some authors (Green & Fleischer, 1964; Kimelberg *et al.*, 1970; Blaurock, 1972) proposed that a single layer of unchanged cytochrome molecules intercalates in the adjacent bilayers.

However, on the basis of the electron-microscopy data, Stoeckenius (1970) came to the conclusion that cytochrome *c* molecules cannot be intact in such a system and should exist in a strongly unfolded state.

The aldehyde fixation spares only lipoprotein (but not pure lipid) structures. The presence of gaps in the hydrophilic regions of aldehyde-fixed multilamellar Lpm systems was detected in this work with the use of electron-microscopy. These facts may indicate that in each gap cytochrome *c* molecules are localized on both sides of the adjacent bilayers. Therefore, two layers of cytochrome *c* molecules should be localized between the adjacent lipid bilayers. Taking into consideration these facts and also the parameters of the multilamellar structure of the Lpm membranes fixed by aldehydes[1] one may assume that molecules of cytochrome *c* do not remain globular in this system but become "deformed."

Some preliminary results have been obtained with the use of infrared spectroscopy and deuterosubstitution studies. It was shown that after interaction of cytochrome *c* with lipids in water, marked structural alterations occur involving ordering of the H-bonds of the peptide chains (Lazarev, Moshkov & Frolova, 1972). It is not excluded that in this case the tertiary structure of the cytochrome molecules may be changed.

Investigations of protein/lipid interaction with the monolayer technique (Kimelberg & Papahadjopoulos, 1971) and some physical methods (Cherry, Berger & Chapman, 1971) also indicate that significant structural changes of protein molecules may take place. This effect may be comparable with that of 6 M urea on the structure of protein molecules (Colacicco & Buckelew, 1971).

In the case of vesicles bound to a single Lpm membrane the thickness (90 to 95 Å) of triple-layered structures (as compared with repeat distance of 85 Å of the multilamellar structures) may be conditioned by a less pronounced "deformation" of the protein molecules localized on both sides of the lipid bilayer. It is not excluded either that in this case on both surfaces of a single lipid bilayer two protein layers are localized: the first, "unfolded" and the second, partially "deformed" layer of protein molecules (Khaiat & Miller, 1969; Quinn & Dawson, 1969; 1970).

The formation of the hexagonal structure in the hydrated isooctane-soluble proteolipid complex was first described by us (Borovjagin & Moshkov, 1970) based on electron-microscopy data. The formation of this phase seems to be determined by the quantity of water in this system.

The lamellar phase of this complex, as revealed by osmium tetroxide fixation, was first described by Sun and Crane (1969).

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1 We took into consideration only the cross-section parameters of the model membranes fixed by aldehydes because, as generally accepted, the osmium tetroxide is a strong protein denaturing agent (Trump & Ericsson, 1965; Lenard & Singer, 1968).

In our experiments the thickness of a single lamella or the repeat distance of multilayered structures after aldehyde fixation were always about 75 to 77 Å. This fact and also the twofold decrease in the amount of phospholipids bound with one protein molecule in the Plm membranes may indicate that in this case a more specific protein-lipid interaction takes place.

The preservation of the fine structure in Lpm and Plm membranes after acetone treatment and aldehyde post-fixation seems to be due to the three-dimensional cross-linkage of the protein molecules through the lipid bilayer by aldehyde polymers. This point of view may be supported by the following facts: (a) the integrity of ordered structure is strongly affected by the nature of aldehydes, the temperature and duration of fixation; (b) after the "A" step, acetone extraction and a short time aldehyde post-fixation the preservation of the lamellar structures was much better than in the control samples fixed in the same conditions. It is not impossible that in the case of the Lpm membranes, remaining phospholipids (about 12%) also promote the integrity of structure. In fact, when about 20% of phospholipids remain in the Lpm membranes the lamellar structures are not affected by osmium post-fixation. It is clear that in the latter case (as in the case of the Plm membranes) the integrity of lamellar structure is maintained at the expense of polymerization of phospholipids by osmium tetroxide (Korn, 1967). It is interesting to note that the triple-layered structure of rat liver submitochondrial particles (SMP) becomes obscure after removal of 72% phospholipids followed by osmium tetroxide fixation (Hall & Crane, 1971).

In similar experiments with rat liver SMP we had the same result, but after acetone extraction glutaraldehyde post-fixations were used the triple-layered structures of SMP were preserved. This fact seems to be due to the destructive effect of osmium tetroxide on proteins, as the structural integrity of SMP seems to be conditioned by proteins rather than by lipid components.

The partial conservation of lamellae in the unfixed Lpm membranes after acetone dehydration followed by Epon embedding may depend on the effects of "acetone fixation" of the protein layers and not on the high percentage of extracted phospholipids.

The results of the experiments carried out with aldehyde pre-fixed model membranes followed by organic treatments according to Napolitano (Napolitano *et al.*, 1967) have shown that both the remaining cephalines and the aldehyde linkage of cytochrome through lipid bilayers stabilized the structure of model membranes. The latter assumption may be further confirmed by our experiments showing that after heating of the model membranes at 70 to 80 °C followed by aldehyde fixation at the same temperatures, marked

increase in the width of their hydrophobic (central) zones occur (Borovjagin *et al.*, 1972). It is of interest that the same results have been obtained with frog myelin sheaths (our own unpublished data) and frog retinal photoreceptor membranes (Borovjagin, Ivanina & Moshkov, 1973).

Digestion of the Lpm membranes with pronase shows that cytochrome *c* molecules in this type of model membrane are localized on the two exterior faces of the lipid bilayer and are not protected by interaction with phospholipids. On the another hand, such localization of cytochrome *c* does not allow phospholipid hydrolysis by phospholipase C (Lenaz *et al.*, 1972). An analogous localization of a considerable part of protein components seems to take place in some biological membranes. For example, pronase digestion of inner mitochondrial membranes (Borovjagin, Moshkov, Khabibullina & Shchipakin, 1971) and membranes of *Mycoplasma l* (Morowitz & Terry, 1969) removes 65 to 80% of membrane proteins and simultaneously releases pure lipid bilayer structures.

The aldehyde post-fixation of the Plm membranes after the same pronase treatment preserves the single- or double- and triple-layered proteolipid structures. These artificial membranes had the same appearance as those of the control samples and their thickness did not change noticeably. The results obtained as a result of the morphological, pronase and acetone treatment experiments with this type of artificial membrane suggest the possibility that cytochrome *c* molecules are buried, totally or in part, within an interphase region on each side of the lipid bilayer.

This study also shows that aldehyde fixations and subsequent embedding procedures can be used as a test showing whether or not real lipoprotein (or proteolipid) complexes form after interaction of lipid bilayers with protein molecules.

Note Added in Proof. Recently, the freeze-fracture technique has been used by us in the study of the structure of both types of model membranes. All the surfaces (hydrophilic and hydrophobic) of the investigated model membranes were relatively smooth. The hydrophilic (etch) surfaces of the proteolipid membranes were somewhat rough.

No significant structural alterations of the cytochrome *c* have been detected by IR spectrometry and deuterosubstitution studies in the hydrated proteolipid membranes.

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