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Simultaneous Measurements of Optical and Electrical Properties of Artificial Membranes Composed of Mitochondrial Lipids and their Interaction with Cytochrome *c*

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Summary. A newly constructed cell, which allows simultaneous measurements of optical and electrical properties, was used to study bimolecular black membranes composed of beef heart mitochondrial lipids and their interaction with cytochrome *c*.

The results show that these highly charged membranes are stable only in relatively limited ranges of boundary conditions. In 0.1 *n* KCl their maximum direct current (dc) resistance is 7×10^8 Ohm cm$^2 \pm 10\%$; the series capacity at 1 kHz is 0.43 μF/cm$^2 \pm 3\%$; the entire thickness, determined by optical reflectivity, is 5.8 ± 0.2 nm.

The interaction between oxidized cytochrome *c* and these lipid membranes is primarily of electrostatic nature, and dependent on the presence of highly charged phospholipids, such as diphosphatidyl glycerol (cardiolipin) and phosphatidyl ethanolamine. The attachment of cytochrome *c* maximally causes a 2.5-fold increase in reflectivity, without any noticeable change in the capacity. This leads to a subsequent instability of the membrane (i.e., rupture) preceded by a rapid increase of the dc conductivity. This behavior is far less pronounced with reduced cytochrome *c*.

Ever since their introduction as a model system for biological membranes (Mueller, Rudin, Ti Tien & Wescott, 1962), black lipid films have proven to be a valuable means for the examination of interactions between lipids and proteins in membranes. In connection with such experiments, two points appeared to need further investigation:

1. Although the methods for determining the physical properties of black lipid membranes (such as dc and ac measurements, determination of optical reflectivity, and spectral absorption) are by no means equivalent, but rather complement one another, it has not been attempted to execute as many of these measurements as possible simultaneously

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on the same film. In this way, many inconsistencies in the interpretation of black film experiments might have been avoided.

The reason for this gap may be that measuring devices designed for electrical measurements are not from the outset suitable for optical measurements and vice versa, or, in other words, simultaneous measurements of electrical and optical properties are, in part, not feasible for technical reasons. Therefore, a universal measuring device was constructed, which enables one to perform various physical measurements simultaneously on the same lipid membrane. The device has been described in detail elsewhere (Nöll, 1976).

2. Especially in recombination experiments with lipids and proteins, materials have often been used which do not interact in membranes *in vivo*.

For this reason the cell devised by us was used to examine the properties of black lipid membranes, which in their composition very nearly reflect the conditions found in the inner membrane of mitochondria extracted from beef heart muscle. The interaction of such lipid membranes with cytochrome *c*, a protein found also in the inner mitochondrial membrane, was subsequently investigated more closely. Although a number of articles concerning this latter complex of questions has already been published (Reich & Wainio, 1961*a*; Reich & Wainio, 1961*b*; Fleischer, Brierley, Klouwen & Slautterback, 1962; Das & Crane, 1964; Das, Haak & Crane, 1965; Kimelberg, Lee, Claude & Mrena, 1970; Dawson & Quinn, 1971; Steinemann & Läuger, 1971; Gitler & Montal, 1972*a*; Gitler & Montal, 1972*b*), it appeared worthwhile to reconsider some of the questions involved in the light of the two points mentioned above.

Materials and Methods

The Lipid System

Mitochondria were prepared based on a method described by Person, Zipper and Felton (1969), whereas the lipid extraction was carried out according to Folch, Lees and Stanley (1957). Necessary care was taken to avoid any unwanted alterations of the material during the preparation. Checks of the mitochondria preparation by electronmicroscopy showed little contaminations from other cell particles, which could not be avoided without recourse to a more refined technique. A slight modification in the composition of the lipid raw extract may be attributable to these impurities, since thin-layer chromatography and the analysis of the lipid phosphorus (according to Zöllner and Eberhagen, 1965) showed the following distribution pattern of the lipid components: the neutral lipid amounts

to 5 to 10% (max.); relative amounts of[1] *DPG*:*PE*:*PC*=1:1.7:2.2 (as compared to about 1:2:2 according to Fleischer, Rouser, Fleischer, Casu and Kritchevsky, 1967). Furthermore, traces of *LPE* and *LPC* as well as *Sph* were found. In order to approach the conditions in the inner mitochondrial membrane as closely as possible, in the recombination experiments the original proportions of *DPG*:*PE*:*PC* were corrected to 1:2:2, and the neutral fat portion was removed (Ernster and Kuylenstierna, 1970). As soon as changes in the chromatogram or in the behavior of the membrane were noticed, a new lipid preparation was used. To produce membranes by the brush technique, the lipids were dissolved in *n*-decane or *n*-tetradecane. Usually, concentrations of 3% proved to be most convenient. The oxidized cytochrome *c* was purchased from Serva. It was reduced by Na-dithionite, then dialysed for 16 hours at 4 °C against 10^3 times the corresponding amount of tris buffer (10^{-2} M, pH 7). The buffer solution was changed twice. Afterwards no oxidized cytochrome *c* could be detected (using the method described by Paul, 1947).

The Measuring Apparatus

The cell device for the combined measurements of electrical and optical properties of black lipid membranes is described in detail elsewhere. A picture of this cell can be seen in Fig. 1*a* and *b*. The actual cell volume was 15 ml. The membrane diameters were generally 5 mm, and the temperature of the cell solution was usually 22 °C. The membrane thickness was derived from the optical reflectivity, according to Cherry and Chapman (1969*a*). The refractive index of the surrounding solution was varied by adding various amounts of sucrose. The pH of the cell solution was adjusted by adding HCl, KOH or NaOH: no buffer was used. Before and after each experiment the pH was checked with a pH-meter.

Results

Properties of Mitochondrial Lipid Membranes

By trying to limit the stability range of the lipid mixture specified above as dependent on KCl concentration and pH, the relationship illustrated in Fig. 2 is found: film formation is basically possible between pH 3 and 9 and at concentrations between 10^{-4} and 10^{-1} *n*. At higher pH values and concentrations film formation becomes critical. In the double hatched region of Fig. 2—an essentially physiological range of pH and ionic strength—the films may be turned black with application of voltage. Using a $CaCl_2$ solution instead of KCl, the double hatched region can be extended to a $CaCl_2$ concentration of 10^{-4} *n*.

In a large number of experiments with this lipid system a well-reproducible maximum dc resistance of 7×10^8 Ohm cm^2 was found in 0.1 *n*

[1] Abbreviations used: *DPG*, diphosphatidyl glycerol (cardiolipin); *LPC*, lysophosphatidyl choline; *LPE*, lysophosphatidyl ethanolamine; *NL*, neutral lipids; *PC*, phosphatidyl choline (lecithin); *PE*, phosphatidyl ethanolamine; *Sph*, sphingomyelin.

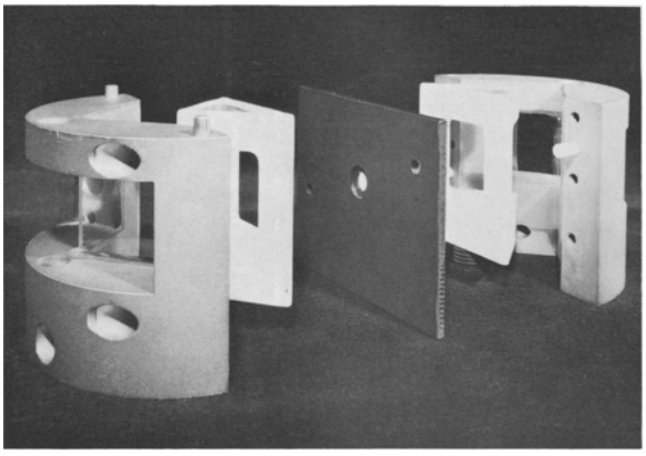

Fig. 1 *a*. Disassembled cell device as used for simultaneous measurements of electrical and optical properties of artificial lipid membranes

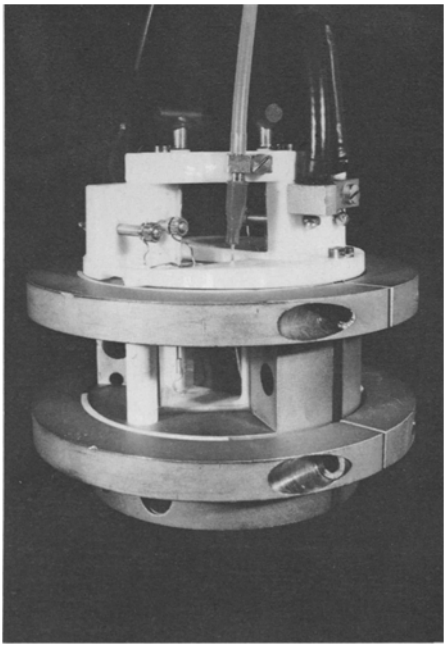

Fig. 1 *b*. Completely assembled cell

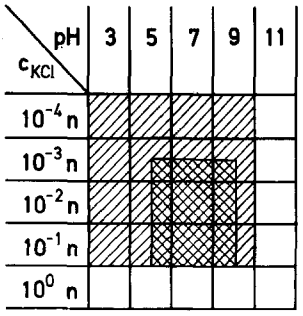

Fig. 2. Stability ranges of artificial lipid membranes from mitochondria in KCl solution: simple hatched area, formation of membranes possible; double hatched area, formation of stable black films possible

KCl solution at pH 7. This is in contrast to frequent reports that the dc resistance of black lipid membranes should be only poorly reproducible, fluctuating over several orders of magnitude. By comparing the electrical and optical behavior of the membranes from the beginning of their thinning until they have turned completely black, it was clearly shown (results not presented here) that any fluctuations in dc resistance are not due to the properties of the lipid material, but are evidently artefacts caused by the teflon aperture in which the films are produced. This finding is especially important with regard to recombination experiments. Even pure and unmodified lipid membranes may show stepwise (even "unit-channel") fluctuations in dc when the hole in the teflon partition is not prepared properly. Under the conditions specified above, and in the frequency range from 50 Hz to 50 kHz, the specific membrane impedance may be represented by a series connection of a virtually frequency-independent ohmic resistance and a constant capacity of $0.43\ \mu F/cm^2 \pm 3\%$.

The determination of the overall film thickness from its optical reflectivity is based on the method of Cherry and Chapman (1969*a*). The variation of the refractive index of the surrounding solution was accomplished by the addition of sucrose (up to 40%). The square root of the film reflectivity is shown in Fig. 3*a* and *b* as dependent on n_o and $n_o - \Delta$. From this data a total thickness of 5.8 nm $\pm$ 3% can be calculated.

It was possible to show, by simultaneously measuring film capacity and optical reflectivity, that the further increase in capacity of lipid films after their turning black (as already observed by White (1970) and others) is indeed accompanied by a further decrease in thickness.

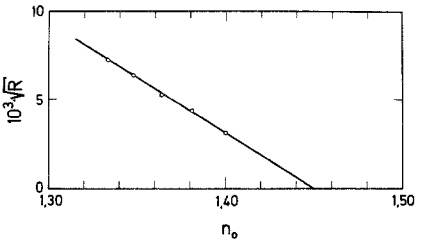

Fig. 3*a*. Variation of film reflectivity with refractive index of surrounding medium

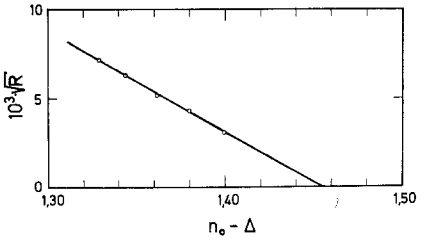

Fig. 3*b*. Variation of film reflectivity with $n_o - \Delta$

This decrease in thickness until the film becomes stationary, determined by capacity and reflectivity measurements, is the same in both cases. This means that it is actually the apolar central layer of the film which decreases in thickness, even after the film has turned black. Therefore, it must be concluded that lipid solution is still flowing out of the central lamella into the film torus, until an equilibrium is reached. In the recombination experiments with cytochrome *c* described below, *stationary* films were used exclusively.

Recombination Experiments with Oxidized and Reduced Cytochrome c

Fig. 4 shows a typical recombination experiment: After a black film of mitochondrial lipids had become stationary in a 0.01 *n* KCl solution at pH 7.3, 1 mg of cytochrome *c*, as a highly concentrated solution, was injected into the rear half of the cell and stirred (at *A*), in order to avoid additional light absorption by cytochrome *c*. (The refractive index in the cell is not changed by the amount of protein present.) Whereas capacity and direct current remain essentially constant, the reflectivity approaches a new, higher value. In *B* the same amount of protein was injected into the front half of the cell. At this point the

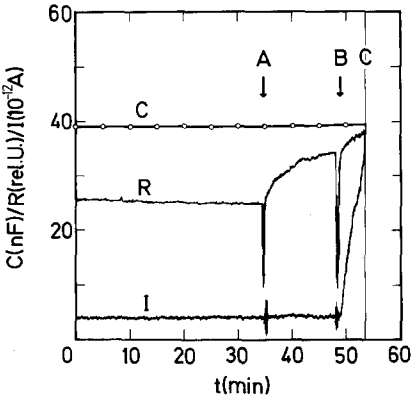

Fig. 4. Synchronous registration of capacity *C*, reflectivity *R* and direct current *I*. *A*: addition of protein and stirring in rear half of the cell. *B*: addition of protein in front half. *C*: film rupture

additional absorption of light by cytochrome *c* has to be taken into account. Although the capacity still remains constant, the reflectivity increases to a value which corresponds to about the same change in reflectivity as after point *A*, whereas the direct current rises very steeply until the film breaks prematurely at point *C*. It is a typical feature of these experiments, that the dc conductivity changes only after addition of protein on both sides of the film, and that the film itself then becomes mechanically very sensitive and unstable, and breaks shortly afterwards. The increase in reflectivity can be controlled to a certain extent by stirring the cell solution more or less vigorously. The steeper the increase in reflectivity, the more short-lived the film. The maximum lifetime of the film in such experiments is about 10 min after adding the protein. The entire reflectivity reaches a maximum of as much as 2.5 times the initial value, without attaining saturation. The reactivity of cytochrome *c* with the lipid film decreases strongly with the concentration of KCl. Between concentrations of 0.01 *n* and 0.05 *n*, a jump is clearly visible (Fig. 5).

Using $CaCl_2$ instead of KCl slows down the reflectivity increase significantly. The reflectivity curves of films in 10^{-3} and 10^{-4} M $CaCl_2$ are shown in Fig. 6. It may clearly be seen that the reaction is faster at lower concentrations of Ca ions. It is noteworthy that stable black films can no longer be formed as soon as cytochrome *c* is added to the cell solution. In this case, the films begin to grow black quickly and spontaneously, but almost always break before they have completely

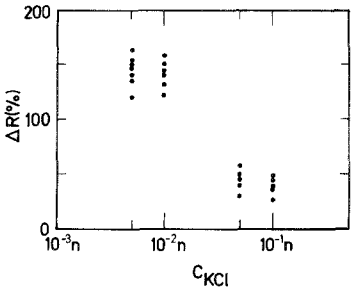

Fig. 5. Maximum reflectivity increase as dependent on concentration of the surrounding KCl solution at pH 7. Equal amounts of cytochrome *c* are added on both sides of the lipid membrane in these experiments

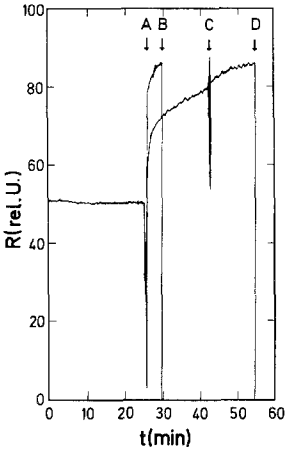

Fig. 6. Increase of reflectivity after addition of equal amounts of cytochrome *c* (at *A*) to a lipid film in 10^{-4} M $CaCl_2$ solution (film breaks at *B*), and in 10^{-3} M $CaCl_2$ solution (film breaks at *D*); *C*: stirring started; pH 7.3

blackened. A recombination of cytochrome *c* with pure lecithin films could not be found.

Using reduced instead of oxidized cytochrome *c*, a clear reduction in the increase of reflectivity, as well as a prolongation in the lifetime of the film by a factor of at least 5, was detected. For comparison, a reflectivity curve, which was obtained with oxidized cytochrome *c* under the same conditions, is shown in Fig. 7. With reduced cytochrome *c* the reflectivity quickly takes on a new constant value, which cannot be significantly increased by further stirring.

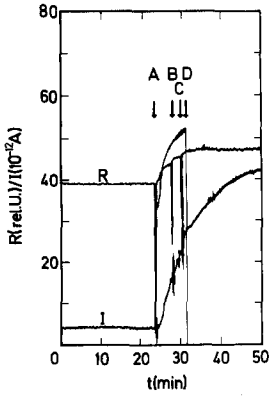

Fig. 7. Increase of reflectivity and direct current after addition of equal amounts of reduced cytochrome *c* on both sides of the film. Reflectivity and current reach new *constant* values, in contrast to a recombination experiment with oxidized cytochrome *c* under the same conditions (film breaks at *D*). *B*, *C*: stirring. Note additional absorption caused by oxidized cytochrome *c* at *A*, which can be neglected in the case of reduced cytochrome *c*

Discussion

From the occurrance of an instability of the black film after the addition of cytochrome *c*, one may conclude that a reaction between the lipid and the protein must have taken place. The optical measurements suggest, moreover, that the protein has become attached to the film surfaces. However, the reflectivity measurements also show that there can be no dense packing of cytochrome *c* on the film surface, because no saturation was observed. This is in contrast to experiments of Steinemann *et al.* (1971). If one takes the same mean refractive index for the two protein layers as for the lipid film, and assuming as they do, that the spherical cytochrome *c* molecule has a diameter of 3 nm, a dense packing on both sides of the film should cause a fourfold increase over the initial value. When one takes into account, however, that the refractive index for cytochrome *c* is higher than that of the lipid film, then a calculation according to Cherry and Chapman (1969*b*) (three layer model) shows that even higher reflectivity values should be expected with densely packed protein layers.

Actually, a maximum increase up to 2.5-fold was measured. Though it cannot be excluded, a deformation of the practically spherical shape of the protein during its attachment seems highly improbable due to

the discrepancies between the theoretical and actual values. Only a determination of the number of cytochrome *c* molecules attached to the film, as determined by absorption measurements (*see* Steinemann *et al.*, 1971), could yield more definite results. Unfortunately, when the present measurements were carried out, a determination of film absorption was not possible for technical reasons, even though the cell used would have permitted such a simultaneous measurement.

The fact that the capacity of the lipid film during the binding process remains virtually constant does not in this case reveal whether, and to what degree, the protein penetrates into the lipid bilayer. Thus, one might well conceive that during this process the thickness of the film and its dielectric properties change in such a way that on the average the total capacity of the membrane remains constant.

The dependency of the reflectivity increases on the ionic strength shows that the interaction is primarily of an electrostatic nature. However, as observed in dc and optical measurements in the case of the asymmetric and the symmetric addition of protein, the marked difference between oxidized and reduced cytochrome *c*, suggests that subsequently other factors might be of importance. Indeed, since the resistance of the film begins to decrease significantly only when cytochrome *c* is added on both sides of the membrane, one could argue that opposite cytochrome *c* molecules would form randomly distributed protein bridges (the diameter of one molecule equals about half the thickness of the unmodified film). When these linkages are sufficiently limited in area compared to the entire area of the membrane and there is a sufficiently high increase in conductivity, this picture would also fully agree with the ac behavior observed. For instance, penetration of cytochrome *c* into lipid monolayers at the air-water interface has been observed by Dawson *et al.* (1971).

On the other hand, this effect is much less pronounced with reduced cytochrome *c*. Therefore, the question arises what the cause for the observed behavior could be. At neutral pH's, oxidized cytochrome *c* differs from the reduced form only in the charge of the Fe ion—a circumstance which does not affect the degree of dissociation of neighboring amino acid side chains (Theorell and Åkesson, 1941). According to Margoliash and Schejter (1966) this implies, however, that the net positive charge of the polypeptide chain would be about 9.5 for both oxidized as well as reduced cytochrome *c*. Therefore, it appears improbable that the observed effect is due to the net charge difference between the two forms of cytochrome *c*. In fact, it seems that the reduction of cytochrome *c* involves conformational changes as well, which would also entail some

rearrangement of the surface charges (Takano, Kalli, Swanson and Dickerson, 1973).

In spite of some uncertainties in the interpretation of the crystallographic data, and the different properties of dissolved *vs.* membrane-bound cytochrome *c*, the oxidized form is obviously more accessible to the outside than the reduced state, which has a more closed and compact conformation. Thus it is conceivable that, upon initial electrostatic attachment, oxidized cytochrome *c* may further penetrate the lipid phase. Since on the other hand, however, the specificity of the positive counter-ions described above plays an important role in the stability of the lipid film, the following explanation cannot be excluded. As metal ions are replaced by oxidized cytochrome *c* on the film surface, regions of instability arise which lead to a gradual weakening of the originally firmly chelated film surface (compare with Papahadjopoulos and Poste, 1975). As long as these defects occur in sufficiently small areas, a higher flow of ions through the resulting micropores should be possible. Only after such channels have reached a critical diameter would a further increase result in a decrease in free surface energy (De Vries, 1958), which finally causes the film to break. In this connection, it is noteworthy that lipid films formed in a cytochrome *c* solution quickly begin to turn black spontaneously, but do not remain stable. This is also in contrast to the experiments of Steinemann *et al.* (1971) with phosphatidylinositide. On the other hand, reduced cytochrome *c*, because of its smaller charge and closer structure, may not be so effective in replacing positive metal ions. The film structure, therefore, remains more stable. In any case, it must be stated that denaturation of cytochrome *c* under these experimental conditions can effectively be excluded since the reduced cytochrome *c* present in the cell is immediately reoxidized by a cytochrome oxidase preparation, according to Person *et al.* (1969).

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