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VOLTAGE-INDUCED CAPACITANCE RELAXATION OF LIPID BILAYER MEMBRANES

EFFECTS OF MEMBRANE COMPOSITION

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SUMMARY

The specific capacity of black lipid membranes of different phospholipids dissolved in *n*-alkanes was investigated. The hydrocarbon thickness of these membranes, as calculated from the electric capacity with a dielectric constant of 2.1, was in most cases close to 5 nm. It was found that the specific capacity is not constant with time after blackening. It shows a linear time dependence characteristic for each lipid/solvent system.

The influence of a transmembrane potential on the capacity of the membranes was measured. It was shown that the extent of the capacity change, obtained 10 s after applying a voltage, was strongly dependent on the lipid composition as well as the solvent content of the membranes. The capacity change of the membranes seems to be caused mainly by a thickness change and not by an area increase of the membranes.

INTRODUCTION

Black lipid membranes, first described by Mueller et al. [1], have been used in the past as models for biological membranes and as tools for physico-chemical studies. The specific membrane capacity C_m is one of its basic properties and it has often been investigated for black films from natural and synthetic lipids dissolved in different *n*-alkanes [2–8]. The thickness of these black films as calculated from capacitance measurements was found to be dependent on the nature of the lipid and the solvent [4, 6, 7, 9]. The small thickness (between 2.5 and 7 nm) together with the results of electron microscope studies [10] make it very likely that the membrane is composed of two monomolecular layers of lipid molecules.

The capacitance and the specific capacity of a black lipid membrane are not constant with time [6, 8, 11]. Caused by a diminishing of the size of the torus surrounding the membrane, the membrane area increases and with this its capacitance [6]. The time dependence of the specific capacity [8, 11] suggests that the black lipid membrane is not in equilibrium. It is likely that the molar ratio of lipid to solvent in

the film is not constant but changes with time. This change in composition may be caused by a disproportionation between lipid and solvent and/or a disappearing of microlenses [12]. In previous papers [7, 11] we showed that the length of the hydrocarbon tail of a lipid has a remarkable influence on the specific capacity. In the following we extend this study to lipids with different polar headgroups, different number and position of the double bond in the hydrocarbon tail, and different types of binding of the hydrocarbon chain to the glycerol residue (ester or ether lipids).

The effect of electric field strength on the specific capacity of a black lipid film has often been studied in the past [5, 6, 12–16]. It has been discussed whether the increase of capacitance by the electric field was due to a thickness decrease or to an area increase of the black film or to both [12–16]. In some cases Young's modulus for the film elasticity has been calculated from the increase in the specific capacity caused by a direct current potential across the membrane [15, 16]. But this analysis has been questioned because of the possible influence of the solvent and the high energy needed to change the distance between adjacent lipid molecules in the membrane [12]. In two recent papers [11, 17] we have shown that the influence of voltage on membrane capacitance depends on the nature of the lipid and the solvent and vanishes for solvent free membranes which have been formed by the Montal-Mueller technique [18]. In this study we investigate more extensively the influence of the torus/membrane ratio on the increase of specific membrane capacity caused by a transmembrane potential as well as the influence of solvent and the time after blackening.

MATERIALS AND METHODS

Most of the phospholipids used in this study were synthesized in our own laboratory (see below). We formed membranes from various L-1,2-diacyl-3-phosphatidylcholines with the following fatty acid residues: phytanoyl (3, 7, 11, 15-$C_{16:4CH_3}$), palmitoleoyl (Δ^9-$C_{16:1}$), oleoyl (Δ^9-$C_{18:1}$), petroselinoyl (Δ^6-$C_{18:1}$), vaccenoyl (Δ^{11}-$C_{18:1}$), elaidinoyl (trans Δ^9-$C_{18:1}$), linoleoyl ($\Delta^{9,12}$-$C_{18:2}$), linolenoyl ($\Delta^{9,12,15}$-$C_{18:3}$), eicosenoyl (Δ^{11}-$C_{20:1}$), arachidonoyl ($\Delta^{5,8,11,14}$-$C_{20:4}$), erucoyl (Δ^{13}-$C_{22:1}$), nervonoyl (Δ^{15}-$C_{24:1}$). Some experiments were performed using a lecithin with mixed chains: L-1-oleoyl-2-stearoyl-3-phosphatidylcholine. An ether phosphatidylcholine (1-*O*-*cis*-9-octadecenyl-2-*O*-hexadecyl-3-phosphatidylcholine) was obtained from Calbiochem. Egg phosphatidylcholine and egg phosphatidylethanolamine were prepared and purified according to ref. 19. DL-Dioleoylphosphatidylethanolamine and the ether analoga as well as 1,2 di-*O*-phytanyl-3-phosphatidic acid were prepared by synthesis (see next section). L-α-Dioleoylphosphatidic acid, bisphosphatidic acid tetraoleoyl and L-α-diarachidinoylphosphatidylcholine were purchased from Serdary and were purified by preparative thin-layer chromatography. After chromatography the lipids had a purity greater than 95 %. The purity of all other lipids was checked by thin-layer chromatography. We found a content greater than 99 %.

The *n*-alkanes (for gas chromatography) and the NaCl (analytical grade) were purchased from Merck. Most of the fatty acids were obtained from Nu Check Prep, Elysian Minn., U.S.A. They were used without further purification; the purity has been checked by gas chromatography. The aqueous solutions of NaCl (10^{-3}–1 M,

in most cases 10^{-1} M) were prepared with twice distilled water. They were unbuffered and had a pH of about 6. Optically black lipid membranes were formed from 0.5 to 2 % (w/v) lipid solutions in different *n*-alkanes [20]. The solution was spread across a circular hole in a vertical Teflon wall separating two aqueous compartments. In most cases the area of the hole was about 2 mm^2. We performed also some experiments with larger holes (approx. 10 mm^2) to ascertain that the membrane area had a neglectible influence on the absolute value of the specific capacity. The smaller hole (area approx. 2 mm^2) was used in order to make the time for the blackening of the membranes small enough. With the smaller hole the blackening time was always less than 5 min. The membranes with a larger area needed a longer time until they were optically black; in these cases the specific capacity immediately after blackening already started with a larger value.

In all experiments the actual area of the black film was measured with an eyepiece micrometer with an estimated error of about 3 %. The torus surrounding the membrane did not exceed 5 % of the total area of the hole, if not otherwise indicated. Most of the measurements were performed at 25 °C (other temperatures are indicated). Bilayer membranes from monolayers (solvent free membranes) were formed as described earlier [11, 18]. A thin plastic septum with a small hole (0.2–0.3 mm in diameter) was clamped between two Teflon troughs, each with a volume of about 3 cm^3. The plastic film was obtained from Yellow Springs Instrument Co (Membrane Kit No. 5937 for oxygen electrodes) and had a thickness of 12.5 μm. The troughs were filled with the aqueous solution; after cleaning of the water surface the two water levels were adjusted below the hole in the septum by means of two syringes. The lipid was then added to the water surface as a 0.1 % (w/v) solution in *n*-hexane. The amount of lipid on the surface was in large excess compared with the amount needed for a monolayer. After the evaporation of the solvent the water level on one side was adjusted just above the hole with the syringe. Then the level on the other side was carefully raised to the same level. The formation of the membrane was monitored by measuring the capacitance (see below). For the measurement of the electrical capacity rectangular voltage pulses of 10 mV with a risetime of 200 ns were applied through Ag/AgCl electrodes to the membranes from a battery-operated pulse generator [11, 17]. The voltage drop which was induced by the capacitive current across an external resistance was measured with a Tektronix 5115 storage oscilloscope. For the external resistance a value between 10 and 50 kΩ was chosen depending on the required sensitivity and time resolution of the experiment. Because of the high membrane resistance ($R_M \approx 10^8\ \Omega \cdot cm^2$) the stationary current I_∞ was about four orders of magnitude smaller as the initial current I_0. In this case the membrane capacity C_m was obtained using the relation $V_m C_m = \tau I_0$ with V_m being the applied voltage, τ the time constant of the exponential decay of the current, and I_0 the current extrapolated to zero time. The values for the capacity obtained by the method were in good agreement with the results obtained from measurements with an alternating current bridge [11, 17].

Synthesis of lipids

The phosphatidylcholines were synthesized according to a slightly modified version of the method of Baer and Buchnea [21]. Fatty acid chlorides were prepared from fatty acid and thionyl chloride. The crude phosphatidylcholine was purified by

chromatography across a silica gel column (Merck Art. No. 7734). By the same method it was also possible to obtain the corresponding lysophosphatidylcholine, for example 1-oleoyllysophosphatidylcholine. A new synthesis using this lysophosphatidylcholine and stearoylchloride gave L-1-oleoyl-2-stearoyl-3-phosphatidylcholine, a lipid with one unsaturated and one saturated C_{18} fatty acid.

The synthesis of DL-1,2-dioleoyl-3-phosphatidylethanolamine was carried out starting from DL-3-tritylglycerol [22] which was acylated with oleoyl chloride to DL-1,2-dioleoyl-3-tritylglycerol. The trityl residue was removed by passing the reaction product through a silicic acid-boric acid column [23, 24]. DL-1,2-dioleoyl-3-phosphatidylethanolamine was obtained by the reaction of DL-1,2-dioleoylglycerol with phthalimidoethylphosphoryldichloride [25] in the presence of triethylamine, saponification of the corresponding chloroderivatives and removal of the phthaloyl residue of DL-*N*-phthaloylphosphatidylethanolamine by hydrazinolysis [26].

The ether analogon DL-1,2-di-*O*-*cis*-9-octadecenyl-3-phosphatidylethanolamine was synthesized by a similar procedure. *O*-Tritylglycerol was alkylated with *cis*-9-octadecenylmesylate in boiling benzene in the presence of KOH. With the product DL-1,2-di-*O*-*cis*-9-octadecenyl-3-tritylglycerol the rest of the synthesis was carried out as described above. DL-1,2-di-*O*-phytanyl-3-phosphatidic acid was synthesized by alkylation of *O*-tritylglycerol with phytanylbromide. The trityl group was then removed with HCl/petrolether. The final product was obtained by phosphorylation of DL-1,2-di-*O*-phytanylglycerol with $POCl_3$.

RESULTS

Absolute value and time dependence of the specific capacity

Black lipid membranes may be regarded as thin layers of low dielectric constant in an aqueous medium with a high dielectric constant. For an approximative description of the thickness d of the hydrocarbon moiety of the membrane the equation for the capacity of a plate condensor may be used:

$$C_m = \varepsilon\varepsilon_0/d \tag{1}$$

C_m is the specific capacity of the membrane, ε the dielectric constant of the membrane and $\varepsilon_0 = 8.85 \cdot 10^{-12}$ F/m the permittivity of free space. In all calculations a value of $\varepsilon = 2.1$, corresponding to the average dielectric constant of a long chain hydrocarbon has been used [6]. The capacity of the polar layers of the membrane is much larger than the capacity of the hydrocarbon core of the membrane. Therefore, d is close to the thickness of the hydrocarbon layer of the membrane. The total thickness of the membrane may be obtained by adding the thickness of the two polar layers (totally 1.0–1.5 nm) to d. The time course of the specific capacity of black lipid membranes of different composition was investigated. The results are given in Fig. 1 and in Tables I and IV. Fig. 1 contains the results obtained from membranes from phosphatidylcholines with mono-unsaturated fatty acid residues of different lengths. The specific capacity falls of more rapidly with increasing chain length for longer chains (24 : 1, 22 : 1 and 20 : 1) than for shorter chains (16 : 1 and 18 : 1). Similar results were obtained for membranes from phosphatidylcholines with saturated fatty acid at 50 °C above the melting point [7]. The asymptotic behaviour of the mem-

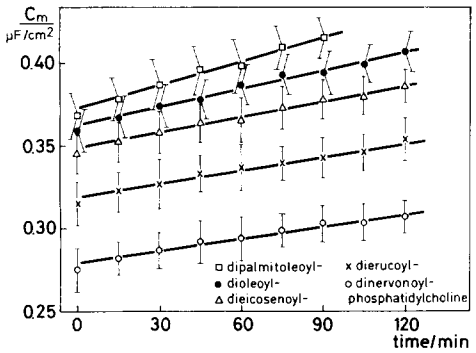

Fig. 1. Time dependence of the specific capacity of membranes from phosphatidylcholines with monounsaturated fatty acid residues of different chain length. The lipids were dissolved in *n*-decane, 0.1 M NaCl, 25 °C. At $t = 0$ the membranes has turned completely black. The experimental points are mean values from at least five membranes. The range of the observed values is indicated by bars. The time dependence of the specific capacity of a single membrane is in most cases similar to the mean value.

TABLE I

SPECIFIC CAPACITY C_m AND INCREASE OF THE CAPACITY $\Delta C_m/C_m$ MEASURED AT MEMBRANES FROM VARIOUS PHOSPHOLIPIDS DISSOLVED IN *n*-DECANE

C_m is the value of the specific capacity 30 min after blackening and d the thickness of the hydrocarbon core of the membrane as calculated from C_m according to Eqn. 1. ρ is the increase of C_m with time measured for at least five membranes during 2 h and is given in percent per h. The pressure p was calculated according to Eqn. 3. The values for $\Delta C_m/C_m$ were obtained from at least four membranes 10 s after application of $V_m = 150$ mV (30 min after blackening). 0.1 M NaCl, 25 °C.

Fatty acid residues	C_m (nF/cm²)	d(nm)	ρ(%/h)	p ($10^3 \cdot Nm^{-2}$)	$\Delta C_m/C_m$
Phosphatidylcholines					
Diphytanoyl (3,7,11,15-$C_{16:4\ CH_3}$)	366±15	5.08±0.21	< 1	8.1	0.17±0.02
Dipalmitoleoyl (Δ^9-$C_{16:1}$)	387±15	4.80±0.18	8.1	9.1	0.34±0.03
Dioleoyl (Δ^9-$C_{18:1}$)	374±13	4.97±0.17	6.1	8.5	0.27±0.03
Dipetroselinoyl (Δ^6-$C_{18:1}$)	399±11	4.66±0.14	< 1	9.6	0.11±0.01
Divaccenoyl (Δ^{11}-$C_{18:1}$)	390±14	4.77±0.18	7.2	9.2	0.26±0.03
Dielaidinoyl (transΔ^9-$C_{18:1}$)	335±13	5.55±0.20	< 1	6.8	0.19±0.02
1-Oleoyl-2-stearoyl	370±15	5.02±0.19	2.5	8.3	0.19±0.02
Dieicosenoyl (Δ^{11}-$C_{20:1}$)	358±13	5.19±0.18	5.2	7.8	0.16±0.02
Dierucoyl (Δ^{13}-$C_{22:1}$)	327±15	5.68±0.24	5.0	6.5	0.09±0.01
Dinervonoyl (Δ^{15}-$C_{24:1}$)	287±11	6.48±0.24	5.4	5.0	0.05±0.005
Egg	339±11	5.48±0.17	8.9	7.0	0.32±0.03
Phosphatidylethanolamines					
Dioleoyl (Δ^9-$C_{18:1}$)	372±12	5.00±0.16	1.9	8.4	0.10±0.01
Egg	328±13	5.67±0.22	< 1	6.5	0.23±0.02
Phosphatidic acid					
Dioleoyl (Δ^9-$C_{18:1}$)	401±14	4.63±0.15	3.8	9.7	0.07±0.01
Bisphosphatidic acid					
Tetraoleoyl (Δ^9-$C_{18:1}$)	328±15	5.63±0.24	–	6.5	0.31±0.03

brane thickness with decreasing chain length may be explained by an increasing or constant and therefore increasing proportion of solvent in the black film. The specific capacity of membranes from the different phosphatidylcholines was found to be more or less dependent on time after blackening. 5–10 min after the membranes have turned completely black (blackening time $\leqq$ 5 min) a strong increase was observed (2–4 %). The subsequent increase of specific capacity with time was dependent on the kind of the fatty acid residue of the phosphatidylcholines. For *cis*-monounsaturated fatty acids (except petroselinic acid) a linear increase of specific capacity with time was found (Table I). The relative increase varied between 5.0 %/h for erucic acid and 8.1 %/h for dipalmitoleic acid. Similar results were obtained for the phosphatidylcholine with mixed chains and for egg phosphatidylcholine. This natural lipid contains mostly fatty acids ($\approx$ 98 %) with 16 or 18 carbon atoms [27]. For certain other lipids the influence of time on specific capacity was small. For membranes from phosphatidylcholine with phytanic, petroselinic and elaidic acid residues the relative capacity increase with time was close to zero. Only a small increase was observed also for membranes from the different phosphatidylethanolamines. The results indicate that for membranes from fluid lipids the lipid/solvent ratio is not constant and changes with time. For a lipid with a branched hydrocarbon tail (diphytanoyl phosphatidylcholine) or with fatty acid residues with a higher melting point (petroselinic acid 33 °C, elaidic acid 44.5 °C, as compared with oleic acid 13 °C and vaccenic acid 13 °C [28]) the lipid/solvent ratio in the membrane remains constant with time or changes only little. Phosphatidylcholines and phosphatidylethanolamines with the same saturated fatty acid residue have phase transition temperatures which are much higher for the phosphatidylethanolamine [29]. This different behaviour indicates that the hydrocarbon core of membranes from a phosphatidylethanolamine may be less fluid than that of membranes from phosphatidylcholine with the same fatty acid residues. This may explain the smaller rate of change of specific capacity with time in the case of membranes from phosphatidylethanolamine.

The specific capacity of membranes from dioleoyl phosphatidylcholine dissolved in differencent *n*-alkanes and of solvent free membranes is given in Table II. The thickness of the hydrocarbon core of the membrane decreased with increasing

TABLE II

INFLUENCE OF THE SOLVENT ON THE SPECIFIC CAPACITY C_m AND ON THE INCREASE OF THE CAPACITY $\Delta C_m/C_m$ CAUSED BY THE INFLUENCE OF A VOLTAGE OF 150 mV MEASURED AT MEMBRANES FROM DIOLEOYLPHOSPHATIDYLCHOLINE

The results were obtained 30 min after the blackening of the membranes from at least eight (C_m) or four ($\Delta C_m/C_m$) membranes, 0.1 M NaCl, 25 °C. The thickness d and the pressure p were calculated according to Eqns. 1 and 3. $\Delta C_m/C_m$ was measured 10 s after the application of the voltage.

Solvent	C_m (nF/cm^2)	d(nm)	p ($10^3 \cdot Nm^{-2}$)	$\Delta C_m/C_m$
n-Octane	377±20	4.93±0.24	–	–
n-Decane	374±13	4.97±0.17	8.5	0.27±0.03
n-Dodecane	422±14	4.40±0.14	10.8	0.21±0.02
n-Tetradecane	486±15	3.82±0.11	14.3	0.12±0.01
n-Hexadecane	624±11	2.98±0.06	23.6	0.04±0.005
Solvent free	728±19	2.58±0.08	31.5	< 0.01

chain length of the solvent. This is consistent with the findings with membranes from egg phosphatidylcholine [6] and from monoolein [6, 16] dissolved in different *n*-alkanes. The change in specific capacity is caused presumably by a decreasing solvent content of the membranes with increasing chain length of the *n*-alkanes. Because of this lower solvent content of the membranes the time dependence of specific capacity should be smaller for dodecane, tetradecane and hexadecane as compared with decane, but the stability of these membranes was poor. The short lifetime of the membranes allowed only the estimation of an upper limit for the increase of the specific capacity of less than 1 % per 30 min. The same estimate applies to solvent free membranes. For this type of membranes the specific capacity was found to be about 16 % higher than for membranes from dioleoylphosphatidylcholine dissolved in *n*-hexadecane.

TABLE III

SPECIFIC CAPACITY C_m OF BLACK LIPID MEMBRANES MADE FROM PHOSPHATIDYLCHOLINES WITH FATTY ACID RESIDUES WITH DIFFERENT DEGREE OF UNSATURATION

The lipids were dissolved in *n*-decane. The specific capacity was measured 30 min after blackening of the membranes. The thickness *d* as calculated from the specific capacity according Eqn. 1 is given. The aqueous phase contained 0.1 M NaCl, the temperature was 25 °C. Each value was obtained from at least eight membranes.

Fatty acid residues	C_m (nF/cm^2)	*d*(nm)
Dioleoyl (Δ^9-$C_{18:1}$)	374±13	4.97±0.17
Dilinoleoyl ($\Delta^{9,12}$-$C_{18:2}$)	416±14	4.47±0.14
Dilinolenoyl ($\Delta^{9,12,15}$-$C_{18:3}$)	582±18	3.19±0.11
Dieicosenoyl (Δ^{11}-$C_{20:1}$)	358±13	5.19±0.18
Diarachidonoyl ($\Delta^{5,8,11,14}$-$C_{20:4}$)	443±14	4.20±0.13

Table III contains the results for membranes from phosphatidylcholines with fatty acid residues of different degree of unsaturation. The specific capacity increased with the number of isolated double bonds in a C_{18} chain. Similar results were obtained for fatty acids residues with a C_{20} chain. The reason for this strong influence of the number of double bonds in the fatty acid residues is not clear. The degree of coiling of single chains may increase with the number of double bonds, so that the space between the single lipid molecules in the membrane is partly filled up by the hydrocarbon chains and not by solvent molecules.

Some experiments were performed with ether lipids. The results were given in Table IV together with the results obtained from membranes composed of lipids with acyl binding of the hydrocarbon tails. One can see from Table IV that the differences between membranes from both types of lipids are small, although the increase of

TABLE IV

SPECIFIC CAPACITY C_m AND INCREASE OF THE CAPACITY $\Delta C_m/C_m$ MEASURED AT MEMBRANES FROM PHOSPHOLIPIDS WITH ESTER OR ETHER BINDING OF THE HYDROCARBON TAILS

The lipids were dissolved in *n*-decane. C_m is the value of the specific capacity 30 min after blackening and *d* the thickness of the hydrocarbon core of the membrane as calculated from C_m according to Eqn. 1. ρ is the increase of C_m with time observed for at least five membranes during 2 h and is given in percent per h. The pressure *p* was calculated according to Eqn. 3. The values for $\Delta C_m/C_m$ were obtained from at least four membranes 10 s after application of V_m = 150 mV (30 min after blackening), 0.1 M NaCl, 25 °C.

Lipid	C_m (nF/cm^2)	*d*(nm)	ρ (%/h)	p ($10^3 \cdot Nm^{-2}$)	$\Delta C_m/C_m$
1-Oleoyl-2-stearoyl-3-phosphatidylcholine	370±15	5.02±0.19	2.5	8.3	0.19±0.02
1-*O*-*cis*-9-octadecenyl-2-*O*-hecadyl-3-phosphatidylcholine	352±13	5.28±0.20	9.9	7.5	0.18±0.02
Dioleoylphosphatidyl-ethanolamine	372±12	5.00±0.16	1.9	8.4	0.09±0.01
1,2-Di-*O*-*cis*-9-octadecenyl-3-phosphatidyl-ethanolamine	357± 9	5.21±0.13	< 1	7.7	0.09±0.01
1,2-di-*O*-phytanyl-3-phosphatidic acid	488±13	3.81±0.10	–	14.4	0.07±0.007

the specific capacity with time for membranes from 1-*O*-*cis*-9-octadecenyl-2-*O*-hexadecyl-3-phosphatidylcholine was the largest measured during all experiments.

All results presented in Fig. 1 and Tables I-IV were obtained at 25 °C and with 0.1 M NaCl in the aqueous phase. The influence of ionic strength on the specific capacity was tested for membranes from dioleoylphosphatidylcholine and from dioleoylphosphatidylethanolamine. We obtained the following values for the specific capacity of membranes at 25 °C from dioleoyl-phosphatidylcholine dissolved in *n*-decane 30 min after blackening: 378 nF/cm^2 for 1 M NaCl, 374 nF/cm^2 for 0.1 M NaCl and 380 nF/cm^2 for 0.01 M NaCl. The corresponding values for membranes from dioleoylphosphatidylethanolamine dissolved in *n*-decane were 368 nF/cm^2 for 1 M NaCl, 372 nF/cm^2 for 0.1 M NaCl and 376 nF/cm^2 for 0.01 M NaCl. The differences between the single values are not significant, therefore the results are consistent with theoretical expectations on the influence of ionic strength on the specific capacity of membranes with zero surface charge [5, 30]. The influence of temperature on the specific capacity of membranes from dioleoylphosphatidylcholine was small. With 0.1 M NaCl and a temperature of 10 °C we obtained a value of 394 nF/cm^2 for the specific capacity 30 min after the membranes have turned black. Under the same conditions but at 40 °C the value for the specific capacity was 350 nF/cm^2. A similar temperature dependence of the specific capacity was found for membranes from oxidized cholesterol [13] and for membranes from dioleoylphosphatidylcholine in 1 M NaCl [17].

Capacity change caused by a transmembrane potential

The observation that the specific capacity of a bilayer increases under the influence of a transmembrane potential V_m has often been discussed in the past [6, 11–13, 31, 32]. It was found that the capacity change was roughly proportional to V_m^2. This may be explained by the action of a pressure on the surfaces of the membranes. The pressure p on the dielectric of a plate condensor is given by the expression:

$$p = \frac{\varepsilon \varepsilon_0}{2d^2} V_m^2 \tag{2}$$

(with the notations of Eqn. 1)
Together with Eqn. 1 one obtains:

$$p = \frac{(C_m V_m)^2}{2\varepsilon \varepsilon_0} \tag{3}$$

The pressure is proportional to the square of the product of specific capacity C_m times the transmembrane potential V_m. The range of validity of Eqn. 3 is small. The specific capacity has to be constant and not dependent on V_m. This is not given in most cases discussed below. However, the use of Eqn. 3 may give some feeling for the force per unit area before the capacity of a membrane was changed by the influence of a voltage.

The capacity change and its time course after applying a potential was measured with a set up similar to that used for capacity measurements [11, 17]. Rectangular pulses of 150 mV amplitude and variable duration t were applied to the membranes. The intervals between two single pulses were much longer than the pulse duration t. The exponential decays of the current after the rising and the falling phase of voltage pulse were recorded together on the screen of the storage oscilloscope as shown in Fig. 2. The charging and discharging times were always neglectible compared with the pulse duration t. The voltage-induced capacity increase ΔC_m was evaluated by comparing the current vs. time curves for the on and the off phase of each pulse with the length t [17]. From Fig. 2 one can see that the current at the off phase of the pulses

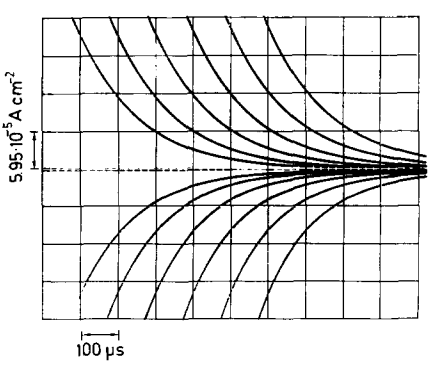

Fig. 2. Turn on (lower curves) and turn off (upper curves) current responses to voltage clamps of −150 mV and varying duration (20 ms, 50 ms, 100 ms, 200 ms, 500 ms and 1 s from left to right) applied to a membrane. The start of the first pulse (20 ms) is at the left border of the record. The trace of each subsequent pulse has been shifted by 100 µs to the right. The membrane was formed from dioleoylphosphatidylcholine dissolved in *n*-decane, the measurement has been performed 30 min after blackening, 0.1 M NaCl, 25 °C.

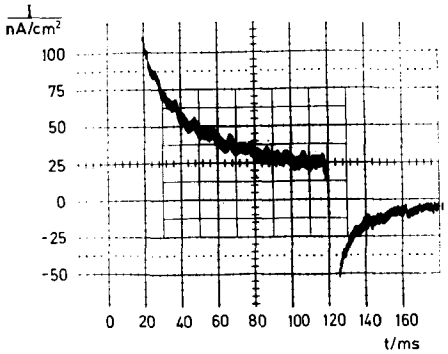

Fig. 3. Turn on and turn off current response of a voltage clamp of 150 mV and about 120 ms duration applied to a membrane from dioleoylphosphatidylcholine dissolved in *n*-decane. The start of the pulse is at $t = 0$. The measurement has been performed 30 min after blackening of the membrane, 0.1 M NaCl, 25 °C. Note the very different scales for time and current of Figs. 2 and 3.

(upper traces in the record) is always larger than the current at the on phase (lower traces). The difference increases with the pulse length t. The capacity change cannot be described by a single exponential function [11, 17].

Fig. 3 shows an experiment performed under identical conditions as in Fig. 2 but recorded with a higher current sensitivity of the oscilloscope and with a slower time deflection. In this case, at very long times, the current response during the on phase of the voltage (150 mV) is much larger than during the off phase (note the very different time scales of Figs. 2 and 3). The difference reflects the current flowing to the membrane after the membrane is already charged to 150 mV. A semi-logarithmic plot of current versus time does not give a straight line. Rather the time course of the current may be represented by a spectrum of relaxation times extending from about 5 ms to more than 100 ms. These findings have to be taken into account in the interpretation of voltage-jump relaxation experiments with bilayer membranes.

Critical for the interpretation of the effects of voltage on the capacitance is the question whether the thickness or the area of the bilayer (or both) are changed under the influence of the electric field strength. Experiments with different torus/black film ratios were performed to separate the influences of both parameters on the time course and magnitude of the capacity change. For membranes with a ratio of torus area to total membrane area of 4, 7 and 12 % the time course of the capacitance change obtained for different pulse durations between 500 μs and 500 ms was approximately the same. From the large values of $\Delta C_m/C_m$ (for instance $\Delta C_m/C_m \cong 0.2$ for dioleoyl phosphatidylcholine/*n*-decane at 500 ms) one can conclude that the capacitance change cannot be caused by an increase of the area alone. Above 500 ms pulse duration the time course of ΔC_m was found to depend on the torus/membrane ratio. With a pulse duration of 10 s membranes with torus/film ratios of 4, 7 and 12 % gave $\Delta C_m/C_m$ values of 27, 29 and 34 %, respectively. Whereas the results for $\Delta C_m/C_m$ obtained with membranes with a small torus (less than 5 % of the total membrane area) are independent of the geometry of the hole in the wall between the two aqueous compartments, the values for $\Delta C_m/C_m$ at large torus/membrane ratios are strongly dependent on the geometrical conditions (for instance, on the length of the bore).

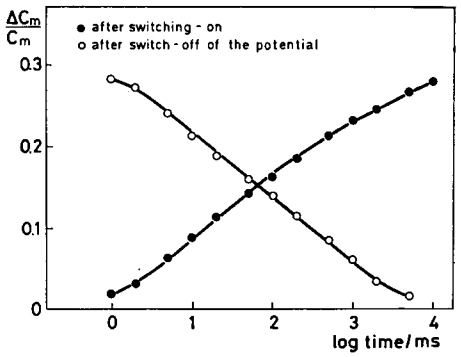

Fig. 4. Time course of the relative capacity change $\Delta C_m/C_m$ for a membrane from dioleoylphosphatidylcholine dissolved in *n*-decane caused by a voltage clamp of 150 mV (full points). The curve with the open points shows the recovery time t of $\Delta C_m/C_m$ after turn off of a voltage clamp of 150 mV and 10 s duration. 0.1 M NaCl, 25 °C.

The reproducibility of $\Delta C_m/C_m$ derived from membranes with a small torus was satisfactory, the scatter of the experimental data obtained from different membranes being within $\pm 10\,\%$. All results given below were obtained from membranes with a small torus to black film ratio (less than 5 %). Similar results were obtained by Requena et al. [12].

An example of the time course of the capacitance measured by the above described method is given in Fig. 4. It contains also the recovery process after the switching-off of the voltage V_m. The experiment was performed in the following way. The falling phase of a pulse with 10 s duration triggered a second pulse generator with test pulses of 10 mV amplitude. The time interval between two pulses was variable between 1 ms and 1 s. The rectangular pulses had a length of 300 μs. The current response to five consecutive pulses was recorded on the screen of the oscilloscope for each time interval. From a logarithmic plot of the current versus time the capacity could be evaluated as described in Materials and Methods. From Fig. 4 one can see that the recovery process of the membrane capacity shows nearly the same time course as the capacity change after switching-on of the voltage.

The influence of longer lasting transmembrane potentials was also tested. It was found that potentials lasting for minutes produced an only partially reversible increase of the capacity of the membranes. The reproducibility of the irreversible increase was very poor. This was also found by Rosen and Sutton [32]. In contrast to this behaviour we found that shorter voltage pulses changed the specific capacity always reversibly. Ten successive pulses of 150 mV voltage and 10 s duration applied to a membrane from dioleoylphosphatidylcholine dissolved in *n*-decane caused no irreversible increase of the specific capacity and the reproducibility for the single values of $\Delta C_m/C_m$ obtained at 10 s was better than 10 %. For these reasons the values for $\Delta C_m/C_m$ were only measured for pulse durations of $\leqq 10$ s. About 85–90 % of the reversible increase of membrane capacity took place during the first 10 s after applying the voltage to the membrane.

Fig. 5 shows the influence of the level of the transmembrane potential on time course and absolute mangitude of $\Delta C_m/C_m$. It was found that at any time t $\Delta C_m/C_m$

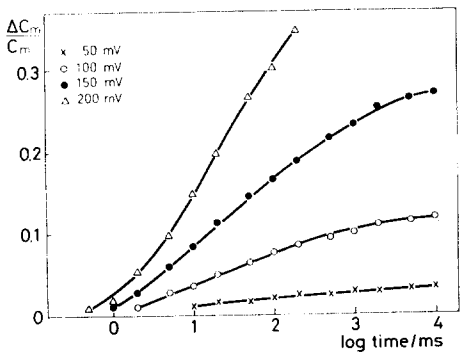

Fig. 5. Time course of the relative capacity change $\Delta C_m/C_m$ for a membrane from dioleoylphosphatidylcholine dissolved in *n*-decane after voltage clamps of different amplitudes. The membrane broke at the upper end of the curve of 200 mV. The aqueous phase contained 0.1 M NaCl, the temperature was 25 °C.

could approximately be represented by a quadratic function of the applied voltage V_m:

$$\Delta C_m/C_m = \alpha V_m^2$$

At $t = 10$ ms, a value of $\alpha = 3.4\ V_m^{-2}$ was found, and at $t = 1$ s, $\alpha = 10\ V_m^{-2}$. These results are similar to those obtained for long times [6, 9, 13] and for $t = 50$ and 500 ms [11]. The proportionality between $\Delta C_m/C_m$ and V_m^2 agrees also with Eqn. 3.

The influence of the age of the membranes on $\Delta C_m/C_m$ is given in Fig. 6. The time course of $\Delta C_m/C_m$ was measured for a membrane from divaccenoylphosphatidylcholine dissolved in *n*-decane and for a membrane from dipetroselinoylphosphatidylcholine dissolved in the same solvent. From Fig. 6 one can see that $\Delta C_m/C_m$ for divaccenoylphosphatidylcholine (upper curves) is strongly dependent on time after blackening of the membrane, thus reflecting the increasing of the specific capacity

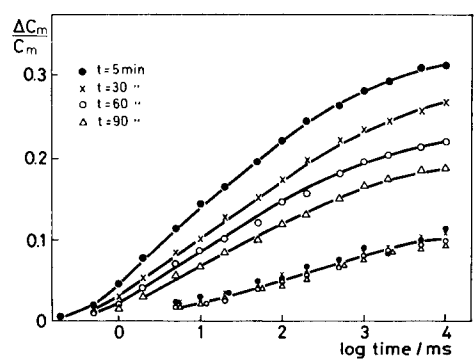

Fig. 6. Time course of the relative capacity change $\Delta C_m/C_m$ after a voltage jump of 150 mV, measured at different times after the blackening of the membrane. The membranes have been formed from divaccenoylphosphatidylcholine (Δ^{11}-$C_{18:1}$, upper curves) or from dipetroselinoylphosphatidylcholine (Δ^6-$C_{18:1}$, lower curve). The lipids were dissolved in *n*-decane, 0.1 M NaCl, $T = 25$ °C.

with time (Table I). For the membrane from petroselinoylphosphatidylcholine only a small influence of the age of the membrane on $\Delta C_m/C_m$ was found. For membranes from this phosphatidylcholine the time dependence of the specific capacity is much smaller. The difference in $\Delta C_m/C_m$ observed with these two lipids may be caused by a different solvent content and/or a different fluidity of the lipids.

Table I summarizes the compressibility of membranes from various lipids dissolved in *n*-decane. Although the pressure on the membrane differs from lipid to lipid only by a factor of less than two, there is a large difference in the $\Delta C_m/C_m$ values (measured 10 s after the application of 150 mV) between the different membranes. For instance, $\Delta C_m/C_m$ is larger by a factor of about 7 for dipalmitoleoylphosphatidylcholine membranes as compared with dinervonoylphosphatidylcholine membranes. The time course of $\Delta C_m/C_m$ for the different lipids is similar (Fig. 7), although for less compressible membranes some kind of delay is visible.

The influence of ionic strength on the compressibility was studied for membranes from dioleoylphosphatidylcholine dissolved in *n*-decane. The variation of $\Delta C_m/C_m$ for NaCl concentrations between 10^{-2} and 1 M was found to be small and lay in the scatter of the experimental data. The temperature dependence of $\Delta C_m/C_m$ was also small. For membranes from dioleoylphosphatidylcholine/*n*-decane in 0.1 M NaCl the $\Delta C_m/C_m$ values (for V_m = 150 mV and 10 s pulse duration) were 0.31 at 40 °C and 0.24 at 10 °C. The time course for 10, 25 and 40 °C was approximately the same although the level of $\Delta C_m/C_m$ at 10 s was slightly different. The specific capacity for these membranes decreased from 10 to 40 °C. A similar correlation between specific capacity and compressibility was found in a series of experiments with membranes made from one and the same lipid. For membranes with a larger specific capacity than the mean value in most cases a smaller value for $\Delta C_m/C_m$ was observed and vice versa.

Fig. 7 shows the influence of chain length of the fatty acid residues on the compressibility of membranes from phosphatidylcholines dissolved in *n*-decane. It is seen that $\Delta C_m/C_m$ strongly decreases with increasing chain of the fatty acid. A similar variation of $\Delta C_m/C_m$ was observed with membranes made from dioleoylphosphatidyl-

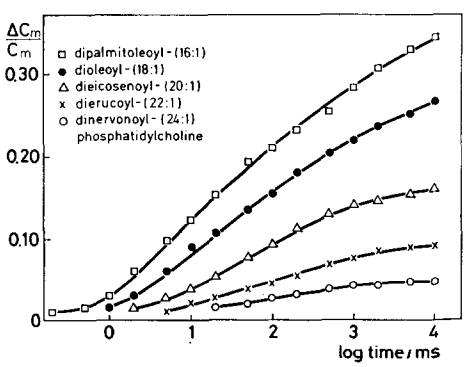

Fig. 7. Time course of the relative capacity change $\Delta C_m/C_m$ for membranes from different phosphatidylcholines dissolved in *n*-decane after a voltage clamp of 150 mV. Each point represents the mean value of experiments obtained from at least four membranes 30 min after blackening, 0.1 M NaCl, T = 25 °C.

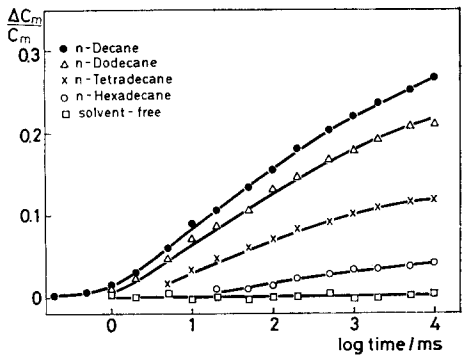

Fig. 8. Influence of the solvent on the time course of the relative capacity change $\Delta C_m/C_m$ for membranes from dioleoylphosphatidylcholine caused by a voltage clamp of 150 mV. Each point represents the mean value of experiments obtained from at least four membranes 30 min after blackening, 0.1 M NaCl, $T = 25$ °C.

choline in different *n*-alkane solvents (Table II and Fig. 8). The compressibility of the membranes decreased in the series *n*-decane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane. With solvent free membranes prepared by the method of Montal and Mueller [11, 18] no change of the membrane capacity under the influence of a transmembrane potential of 150 mV was observed (Fig. 7), although they are perhaps only virtual solvent free and may retain some *n*-hexane [11, 34]. This finding could be extended for a duration of the applied potential of minutes and voltage up to 300 mV. The time course of less compressible membranes shows some kind of delay and seems to be shifted to longer times (Figs. 7 and 8).

The results obtained with membranes from lipids with the same fatty acid residues but different polar head groups are given in Fig. 9. There is a remarkable decrease of $\Delta C_m/C_m$ in the series dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine and dioleoylphosphatidic acid, although the specific capacity of these membranes and, according Eqn. 3, the pressure (Table I) is nearly equal. Fig. 9 contains also results obtained with membranes from natural lipids dissolved in *n*-

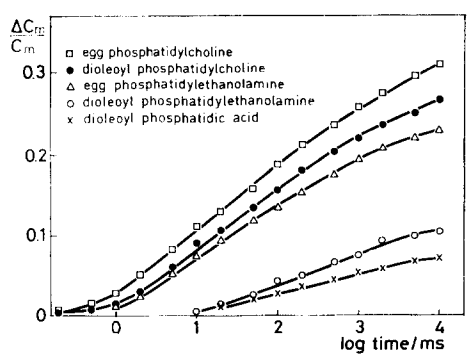

Fig. 9. Time course of relative capacity change $\Delta C_m/C_m$ after a voltage jump of 150 mV for membranes from lipids with different polar headgroups. The lipids were dissolved in *n*-decane. Each point represents the mean value of experiments obtained from at least four membranes 30 min after blackening, 0.1 M NaCl, $T = 25$ °C.

decane. Whereas the time course of $\Delta C_m/C_m$ for synthetic dioleoylphosphatidylcholine and natural egg phosphatidylcholine is almost the same, there is a large difference between synthetic (dioleoyl) and natural (egg) phosphatidylethanolamine. This different behaviour may be caused by the composition of the fatty acid residues of the two natural lipids. Egg phosphatidylcholine contains mainly C_{16} and C_{18} fatty acid chains with presumably one double bond [27] and its fatty acid composition is therefore close to that of synthetic dioleoylphosphatidylcholine. Egg phosphatidylethanolamine contains about 30 % C_{20} and C_{22} fatty acids and about 40 % fatty acids with more than one double bond [33]. This fact may explain the large difference between the compressibility of membranes from natural and synthetic phosphatidylethanolamine.

Table IV contains the results obtained for membranes composed from lipids with ester or ether binding of the hydrocarbon residue dissolved in *n*-decane. It is remarkable that the type of binding of the hydrocarbon tail has only a small influence on the absolute value of $\Delta C_m/C_m$. The same was found for the time course and for the specific capacity.

DISCUSSION

In this study we have investigated the influence of the membrane-forming material (lipid and solvent) on the specific membrane capacity as well as on the rate of capacitance change under the influence of a transmembrane potential. In addition, we have studied the influence of membrane composition on the time course of the steady-state specific capacity. It was found that the fluidity of the lipid indicated by the melting point of the fatty acid residues or by the phase transition observed in lipid/water mixtures changes the time course of the specific capacity whereas the influence on the absolute value is low. The change with time of specific (steady-state) capacity was large for membranes from phosphatidylcholines as compared with membranes from phosphatidylethanolamines with the same fatty acid residues. Likewise, for membranes from phosphatidylcholines with fatty acids of low melting point the increase of capacitance with time was larger than for phosphatidylcholines with high melting point fatty acids or fatty acids with branched chains. The increase with time of the specific capacity was always found to be a nearly linear function of time up to at least 2 h. In most cases this time course continued until the membranes broke. This indicates that the molar ratio of lipid to solvent in these membranes changes with time. The loss of solvent is also visible in the experiments where we measured the capacity change caused by a transmembrane potential as a function of the after time blackening (Fig. 6).

In cases where the steady-state capacitance was constant in time, the membranes were usually stable for longer times and sometimes stayed overnight. Even after many hours, the specific capacity of these membranes was the same within the scatter of the experimental data. Also the value of $\Delta C_m/C_m$ for a transmembrane potential was found to be independent of the time after blackening for this type of membranes (Fig. 6). The reason for the different time dependence of C_m is not clear, but it seems that membranes from lipids with higher melting points or from lipids with a smaller polar headgroup tend to have a constant ratio of lipid to solvent. However, if the results obtained with membranes from phosphatidylcholines with longer

chain fatty acid residues are taken into account, it is seen that the correlation between melting point and time behaviour of C_m is not general. The increase of the specific capacity observed with membranes from dinervonoylphosphatidylcholine or dierucoylphosphatidylcholine shows a similar behaviour as obtained with membranes from dioleoylphosphatidylcholine, although the melting points of the fatty acids are different [28].

The relative capacity change $\Delta C_m/C_m$ caused by a transmembrane potential was found to depend on the nature of lipid and solvent and on time after blackening of the membrane. The results indicate that the capacity change originates from a thinning of the membrane. Because of the low pressure and the high energy involved in thinning a bilayer at constant volume [12] it is not likely that this thinning is caused by the elastic properties of the membranes. The degree by which the membrane thickness changes under the influence of an electric potential seems to be correlated with the solvent content of the membrane. For membranes with little or no solvent the capacity change was found to be small or zero. This is consistent with the finding that membranes with a pronounced time dependence of the steady-state capacity also show a strong voltage dependence of C_m.

A possible explanation for the capacity change caused by a potential difference was given previously [11, 12]. A membrane composed of two monomolecular layers of lipid molecules with a large amount of solvent is presumably not homogeneous and contains small regions of increased solvent content and therefore increased thickness. A potential across the membrane creates a pressure which, according to Eqn. 3, is larger at the thin bilayer parts of the membrane. Because of the fluid nature of the membrane the solvent molecules may be shifted laterally from thinner to thicker parts of the membrane, i.e. from areas with a high pressure to areas with a low pressure. The process is represented schematically in Fig. 10. After some time there must exist large areas where the thickness is considerably smaller than in the rest of the membrane. On the basis of Eqn. 1 it is easily shown that the total capacity increases in this way, if the whole process takes place at constant volume of the film.

The results obtained from membranes with different solvents and the findings with solvent free membranes support this hypothesis. In the series *n*-decane, *n*-dodecane, *n*-tetradecane, *n*-hexadecane and solvent free membranes the specific capacity increases whereas the compressibility decreases simultaneously, indicating a decreasing solvent content. Experiments in the presence of carrier molecules or lipophilic ions with membranes composed of one and the same lipid but different solvents support the view that the structure and the dynamic properties of the membrane are mainly determined by the nature of the lipid and only to a lesser extend by the solvent (Benz, R., in preparation).

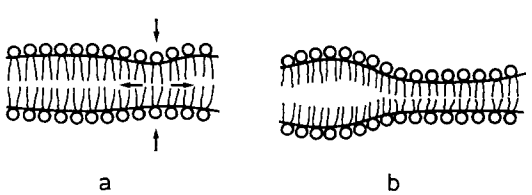

Fig. 10. Effect of pressure caused by a transmembrane potential on a black lipid membrane. (a) Bilayer before and (b) after applying the potential difference.

From the small thickness of solvent free membranes one can conclude that the hydrocarbon chains of the lipids are more or less coiled and this may also be the case in the membranes containing solvent. The rate of lateral diffusion of solvent molecules in the membranes may depend on the molar ratio of solvent to lipid as well as on the fluidity of the lipid. Therefore, the capacity change caused by a transmembrane potential may also be influenced by these parameters.

The time course of $\Delta C_m/C_m$ shows that most of the change takes place within 200 ms for highly compressible membranes, and within about 1 s for less compressible membranes. After switching off of the voltage the capacitance relaxes to the original value within about the same time. This finding indicates that lipid and solvent in the membrane are in a stable quasi-stationary state.

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REFERENCES

1 Mueller, P., Rudin, D. O., Tien, H. T. and Wescott, W. C. (1962) Nature 194, 979–980
2 Andrews, D. M. and Haydon, D. A. (1968) J. Mol. Biol. 32, 149–150
3 Haydon, D. A. (1970) in Permeability and Function of Biological Membranes (Bolis, L., Katchalsky, A., Keynes, R. O., Loewenstein, W. R. and Pethica, B. A., eds.), pp. 185–194, North-Holland Publ. Co., Amsterdam
4 Andrews, D. M., Manev, E. D. and Haydon, D. A. (1971) Spec. Discuss. Faraday Soc. 1, 46–56
5 Läuger, P., Lesslauer, W., Marti, E. and Richter, J. (1967) Biochim. Biophys. Acta 135, 20–32
6 Fettiplace, R., Andrews, D. M. and Haydon, D. A. (1971) J. Membrane Biol. 5, 277–296
7 Stark, G., Benz, R., Pohl, G. W. and Janko, K. (1972) Biochim. Biophys. Acta 266, 603–612
8 White, S. H. and Thompson, T. E. (1973) Biochim. Biophys. Acta 323, 7–22
9 White, S. H. (1974) Biochim. Biophys. Acta 356, 8–16
10 Henn, F. A., Decker, G. L., Greenwalt, J. W. and Thompson, T. E. (1967) J. Mol. Biol. 24, 51–58
11 Benz, R., Fröhlich, O., Läuger, P. and Montal, M. (1975) Biochim. Biophys. Acta 394, 323–334
12 Requena, J., Haydon, D. A. and Hladky, S. B. (1975) Biophys. J. 15, 77–80
13 White, S. H. (1970) Biochim. Biophys. Acta 196, 354–357
14 Wobschall, D. (1972) J. Colloid Interface Sci. 40, 417–423
15 Crowley, J. M. (1973) Biophys. J. 13, 711–724
16 White, S. H. (1975) Biophys. J. 15, 95–117
17 Bamberg, E. and Benz, R. (1976) Biochim. Biophys. Acta 426, 570–580
18 Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3561–3566
19 Singleton, W. S., Gray, M. S., Brown, M. L. and White, J. L. (1965) J. Am. Oil Chem. Soc. 42, 53–56
20 Benz, R., Stark, G., Janko, K. and Lauger, P. (1973) J. Membrane Biol. 14, 339–364
21 Baer, F. and Buchnea, D. (1959) Can. J. Biochem. Physiol. 37, 953–959
22 Jackson, J. E. and Lundberg, W. O. (1963) J. Am. Oil Chem. Soc. 40, 276–278
23 Buchnea, D. (1971) Lipids 6, 734–739
24 Buchnea, D. (1974) Lipids 9, 55–57
25 Hirt, R. and Berchtold, R. (1957) Helv. Chim. Acta 40, 1928–1932
26 Kaplun, A. B., Kabanova, M. A., Lyntik, A. J., Shrets, V. J. and Evstigneeva, R. P. (1973) Zh. Obshch. Khim. 43, 1839–1844
27 Lundberg, B. (1973) Acta Chem. Scand. 27, 3545–3549
28 Wissenschaftliche Tabellen, Documenta Geigy, 7, Auflage Geigy Pharma edn., pp. 362–365

29 Träuble, H. (1971) Naturwissenschaften 58, 277–284
30 Everitt, C. T. and Haydon, D. A. (1968) J. Theoret. Biol. 18, 371–379
31 Babakov, A. V., Ermishkin, L. N. and Liberman, E. A. (1966) Nature 210, 953–955
32 Rosen, D. and Sutton, A. M. (1968) Biochim. Biophys. Acta 163, 226–233
33 Holub, B. Y. and Kuksis, A. (1969) Lipids 4, 466–472
34 White, S. H., Petersen, D. C., Simon, S. and Yafuso, M. (1976) Biophys. J. 16, 481–488