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Voltage Jump/Capacitance Relaxation Studies of Bilayer Structure and Dynamics*

Studies on Oxidized Cholesterol Membranes

D. F. Sargent

Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg, CH-8049 Zürich, Switzerland

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Summary. A voltage-jump technique for the study of the time course of small, voltage-induced changes in the structure of single bilayers is presented, and a method is introduced whereby electromechanical (electrostrictive) phenomena can be separated from dielectric relaxations. As no foreign molecules need be introduced into the bilayers, the question about probe artifacts is eliminated. The time constants and amplitudes of dielectric relaxations in oxidized cholesterol bilayers at 21 °C, along with their tentative identification are: (a) $\tau = 3.3$ msec, $\Delta \mathbf{C}/\mathbf{C}_0 = 0.8$ % and $\tau = 0.7$ msec, $\Delta \mathbf{C}/\mathbf{C}_0 = 0.6$ %: reorientation in the plane of the membrane of domains or clusters of dipoles. (b) $\tau = 155$ μsec, $\Delta \mathbf{C}/\mathbf{C}_0 = 1.5$–3 %: rotational reorientation of individual molecules. (c) $\tau = 18$ μsec, $\Delta \mathbf{C}/\mathbf{C}_0 = 1.4$ %: small amplitude reorientations of individual dipoles about an axis lying in the plane of the membrane. Electrostictive effects with time constants between about 2 and 50 msec were also detected. A temperature study of both the dielectric and electrostrictive phenomena is reported. The application of the technique to other membrane compositions and to a variety of BLM problems is discussed.

The ultimate goal of research using the bilayer model membrane is to explain observed effects on a molecular level. Unfortunately, the minute quantity of material present in planar bilayers puts direct measurement of their molecular architecture beyond the capabilities of most conventional methods. An exception is the work of Yguerabide and Stryer (1971), who showed that fluorescence spectroscopy of macroscopic bilayer vesicles (1–4 mm diameter) was possible. Other spectroscopic studies have been done in special cases where chromophores (e.g., chlorophyll) are incorporated in the BLM (black lipid membrane) (e.g., Cherry, Hsu & Chapman, 1972; Steinemann, Stark & Läuger, 1972). For the most part, however, indirect methods must be used. Stark, Benz, Pohl and Janko

* This work was done in the laboratories of Prof. R. Schwyzer.

(1972) used ion conductivity in the presence of valinomycin as a probe of structural changes in BLM. Another possibility is the study of the bilayer capacitance, which depends on both the make-up and geometry (e.g., thickness) of the membrane (Hanai, Haydon & Taylor, 1965*a*, *b*; Läuger, Lesslauer, Marti & Richter, 1967; Andrews, Manev & Haydon, 1970; White, 1970*b*; Coster & Smith, 1974). This paper reports on the time course of the change in capacitance following changes of the applied voltage.

Theory

A voltage applied across a bilayer can orient naturally occurring dipoles, and will tend to compress the membrane, which may thin out and/or extend as a result (Babakov, Ermishkin & Liberman, 1966; Läuger *et al.*, 1967; White, 1970*a*, *b*; White & Tompson, 1973). The magnitude and time course of these effects will depend on the structure of the bilayer and bulk phases. Such effects will also be reflected by changes in membrane capacitance, so that one might hope to correlate the capacitative changes with structural properties. (Further time-dependent properties attributable to a Maxwell-Wagner dispersion in bilayers have been described by Coster and Smith, 1974.)

The capacitance of a bilayer is given by the formula

$$\mathbf{C}_m \propto \varepsilon_m \cdot \frac{A}{d} \tag{1}$$

where ε_m = dielectric constant of bilayer material, A = area of bilayer region, and d = thickness of bilayer. As an electric field could affect all three parameters, each must be considered separately.

Dielectric Constant

The dielectric constant of the membrane reflects both the dielectric properties of the individual molecules and their organization in the bilayer[1]. Changes in molecular properties are extremely rapid and are not of consequence in the present study. Molecular reorientations, on the other hand, are: both intrinsic dipoles and the axis of highest polarizability of anisotropic molecules will tend to align themselves in the field (Böttcher, 1952). The amplitude and time course of such motions are reflected in the *displacement current* induced in the external circuit. For small field strengths

1 One can usually neglect the contribution from the Plateau-Gibbs border, which generally has a smaller area and much greater average thickness than the bilayer (White, 1972).

the charge displaced is proportional to the scalar product of the dipole moment and the electric field, i.e., it depends on the field direction as well as magnitude. Such behavior will be called "asymmetric" with respect to voltage. For comparison with the next section, we note here that $\mathbf{Q}_d$, the total charge transferred due to dipole reorientation, will be the same for a voltage change of V volts, independent of the initial voltage. Specifically, apart from saturation effects,

$$\mathbf{Q}_d(-V/2 \text{ to } +V/2)=\mathbf{Q}_d(0 \text{ to } +V). \tag{2}$$

In liquids, saturation often starts above 10^5 V/cm. Such field strengths are readily obtainable in BLM, so that saturation effects could be expected at the higher voltages.

Membrane Thickness

The dependence of membrane thickness on applied voltage ("electrostriction") has been described in the literature (e.g., Andrews *et al.*, 1970). The associated changes in capacitance have been found to depend approximately linearly on the square of the applied voltage V so that $\Delta\mathbf{C}_m=\mathbf{Q}_e/V\propto V^2$. Thus $\mathbf{Q}_e$, the charge that flows due to electrostriction, will be given by

$$\mathbf{Q}_e\propto V^3. \tag{3}$$

Note that $\Delta\mathbf{C}_m$ does not depend on the sign of the applied voltage, so that suddenly changing the membrane voltage from $-V/2$ to $+V/2$ should not result in a (further) relaxation. Both dipole reorientation and membrane thinning affect the *specific capacity* of the bilayer, i.e., the corresponding $\mathbf{Q}$'s will be proportional to membrane area.

Membrane Area

The formation of a ring of bilayer outside the original bilayer region due to electrostrictive forces has been described by Babakov *et al.*, (1966) and White and Thompson (1973), among others. As the area change is also proportional to the square of applied voltage (Babakov *et al.*, 1966), no distinction between an area or a thickness change is possible on the basis of voltage-dependence. However, a thickness change will depend approximately linearly on the initial *area*, while an area increase at the border will depend approximately linearly on the *circumference*, at least for small area changes. The relative values of membrane area and circum-

ference can be varied experimentally (e.g., Hanai *et al.*, 1965*b*), so that it should be possible to distinguish between the two mechanisms without resorting to the demanding techniques necessary for direct optical determination of bilayer area.

Materials and Methods

Membranes were formed from cholesterol (Fluka *purum*, recrystallized from ether) oxidized in decane (Fluka *purum*, passed through an alumina column before use) by bubbling with oxygen at 170 °C for about 6 hr (modification of the method of Tien, Carbone and Dawidowicz, 1966). When solvents other than decane were used, a sample of the decane solution was evaporated, and the residue redissolved in the desired solvent. The membrane-forming solutions were saturated in cholesterol. Bilayers were "aged" for at least 15 min under application of 100 mV pulses, thus bringing them to the "final state" described by White (1970*b*).

No differences in results were found between membranes formed in fresh aqueous phases or in solutions in which many membranes had been made and remade over the course of several hours. Thus, as in White's work (1970*b*), the phenomena observed are not affected by the amount of solvent (octane or decane) present in the aqueous phase.

The aqueous phase was 0.2 M NaCl, buffered with 8 mM phosphate (pH = 7), unless otherwise specified. Membranes were formed on a 0.15 cm diameter hole in a teflon cup[2] placed in a glass container. Both units were cleaned with acetone and ethanol, and then rinsed thoroughly with double distilled water. Detergent substances, which could conceivably give rise to adsorption phenomena, were thus avoided.

Lipid was spread either by the brush technique or, when lipid was already present on the cup, by pulling it over the hole with an air bubble blown from a Pasteur pipette. The latter method resulted in a larger ratio of bilayer to border area.

The outer glass container was placed in a temperature-controlled aluminum jacket making tight contact with the bottom and three of its four sides, and the set-up shielded electrically. Measurements were made with a single pair of Ag/AgCl electrodes, immersed to give an area of at least 0.5 cm^2. To show that polarization effects at either the electrode/solution or membrane/solution interfaces were not significant, a trial was made with one electrode contacting the solution only through a film of electrolyte supported by its surface tension. An electrical R-C analog of the membrane was placed in series with the electrodes and measurements made in the normal manner (*see* next paragraph). No time-dependent current changes were observed down to a resolution of better than 10^{-10} A for time constants greater than 20 μsec.

Capacitative relaxation was determined by measuring the time course of the displacement current following a step change in potential. A detailed description of the construction and operation of the apparatus is given elsewhere (Sargent, 1975*a*), but the principle of the technique is presented in Fig. 1. Curves were recorded on a Tektronix 5103N storage oscilloscope and photographed for processing with a Hewlett-Packard 9830A calculator with 9864A digitizer attachment. An example is shown in Fig. 2, where an R-C test circuit having a known "relaxation" was placed in series with the electrodes.

Any capacitative relaxation is assumed to have an exponential time course. After adjusting the analog circuit parameters to balance the charging peak ($R'_m = R_m$, $\mathbf{C}'_m = \mathbf{C}_m$ ($t \simeq 0$)), and for $t > 4\tau_c$ ($\tau_c = \mathbf{R}_s\mathbf{C}_m$ = charging time constant of BLM),

$$I(t) \simeq \sum_i I_o^i e^{-t/\tau_i}. \tag{4}$$

2 Gift from Mr. H. Vögeli, Biochemistry Dept. (Prof. Semenza), ETH Zurich.

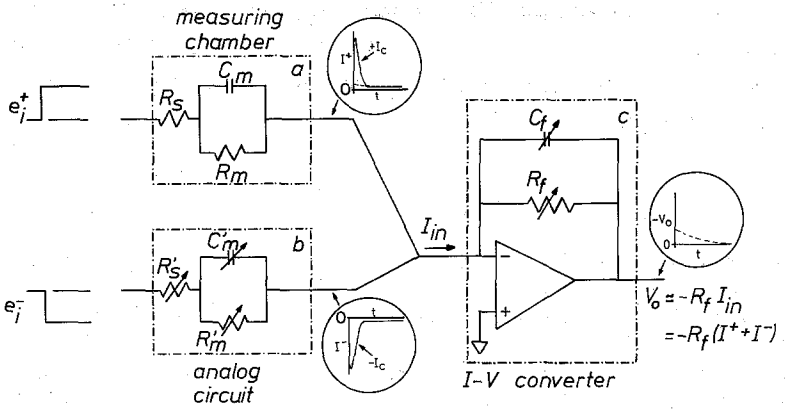

Fig. 1. Outline of the technique used for measurement of current relaxation in BLM following a sudden change in voltage. (*a*) A positive voltage is applied to the electrodes at time $t=0$. This causes a large charging current (I_o) to flow, which decays with a time constant of $\mathbf{R}_s\mathbf{C}_m$, where $\mathbf{R}_s$ = solution + electrode resistance and $\mathbf{C}_m$ = membrane capacitance. In addition, there may be relaxation currents caused by voltage or time-dependent changes in $\mathbf{C}_m$, and a d-c component through $\mathbf{R}_m$. (*b*) At time $t=0$ a negative voltage is applied to an R-C analog circuit which models the parameters of the experimental system ($\mathbf{R}'_s$ and $\mathbf{C}'_m$ are adjusted to match the peak current and the initial decay of the charging peak, respectively; $\mathbf{R}'_m$ can be found by measuring the d-c resistance, $\mathbf{R}_m$, of the bilayer). In this way a current is generated that is equal in magnitude but opposite in sign to that generated by the membrane charging current and the d-c component. The currents from circuits (*a*) and (*b*) are combined, resulting in a cancelling of the charging peaks and d-c currents. The net current is fed into the current-voltage converter (c), which produces a voltage (V_o) proportional to the input current (I_{in}). The gain of the current voltage converter is controlled by the feedback resistor $\mathbf{R}_f$ and the time constant is given by $\mathbf{R}_f\mathbf{C}_f$. In the present study $\mathbf{R}_m$ was always greater than $10^9\,\Omega$, so that the d-c current was less than 10^{-10} A. As this is below the resolution of the apparatus, the d-c component could be neglected

The smallest number of components compatible with the experimental uncertainty was used in all analyses.

The amplitudes are only meaningful when normalized in some manner: as an initial trial, the values were expressed per unit area, of which the simplest measure is the membrane capacity at zero voltage, $\mathbf{C}(0)=\mathbf{C}'_m$. Thus it is convenient to present relaxation amplitude (R.A.) as fractional or per cent changes in capacitance, for which the complete expression is

$$\text{R.A.}^i \equiv \Delta\mathbf{C}_i/\mathbf{C}'_m = I_o^i \cdot \tau_i/(V_o \cdot \mathbf{C}'_m). \tag{5}$$

This is related to the "dielectric increment" through the dielectric constant, but as the latter is not known for all the conditions met, the phenomenological "relaxation amplitude" is used throughout this report.

The resolution of the apparatus allows the detection of $\Delta\mathbf{C}_i$'s of about 1 pF with time constants of several microseconds or about twice τ_c, whichever is larger. At longer time constants the sensitivity increases, so that it is possible to detect changes in membrane capacitance of 0.05 % or less. With 0.2 M NaCl as electrolyte the total series resistance varied between 1165 Ω (16 °C) and 860 Ω (34 °C), giving charging time constants of about 3.3 and 2.5 μsec, respectively (assuming $\mathbf{C}_m \simeq 3$ nF). All relaxation phenomena reported here are considerably slower than this, so that no inaccuracy is introduced from this source.

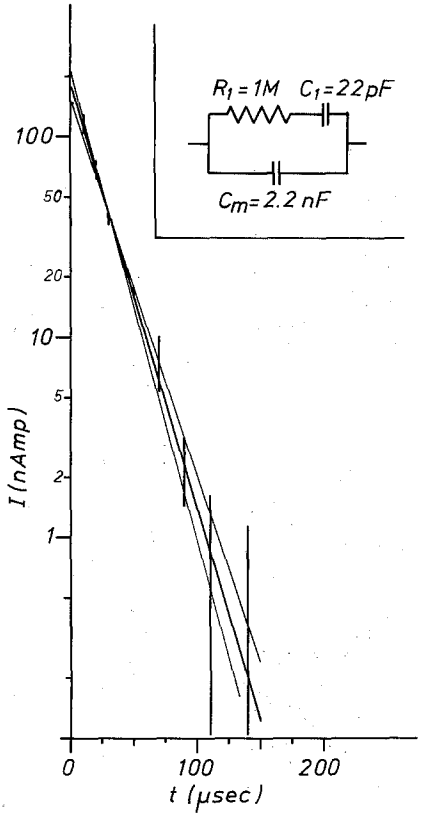

Fig. 2. Test of the relaxation apparatus and effects of diffusion polarization. The test circuit shown in the inset was connected in series with the measuring chamber (*no* membrane present). The electrodes were raised to give a minimum area of contact with the salt solution (0.2 M NaCl) and thus accentuate any diffusion polarization effects. With a solution/electrode resistance of about 1200Ω and an applied voltage of 200 mV the following values hold: charging time constant of $\mathbf{C}_m$ is $\mathbf{R}_s\mathbf{C}_m$=2.65 μsec; charging time constant of $\mathbf{C}_1$ (="relaxation time constant") is $\mathbf{R}_1\mathbf{C}_1$=22 μsec; initial current to be compensated by the analog circuit is $\Delta V/\mathbf{R}_s \simeq 1.6 \times 10^{-4}$ A; initial "relaxation" current is $\Delta V/\mathbf{R}_1 = 2 \times 10^{-7}$ A. Measured values: $\mathbf{R}_1\mathbf{C}_1 = 21 \pm 2$ μsec, $\Delta V/\mathbf{R}_1 = (1.9 \pm 0.3) \times 10^{-7}$ A. Averages and standard deviations of four trials are shown. *Inset:* $\mathbf{M} = 10^6\,\Omega$, nF = 10^{-9} Farad, pF = 10^{-12} Farad

Results

Asymmetrical Effects

A formal description of the asymmetric relaxation currents ($-V/2$ to $+V/2$) required four exponential terms to fit the region between 10 μsec and 10 msec to within the experimental accuracy. Fortunately, the relative amplitudes of the components is such that even moderate uncertainties in the slower components have only a minimal effect on the analysis of the faster components. Nonexponential processes cannot be excluded

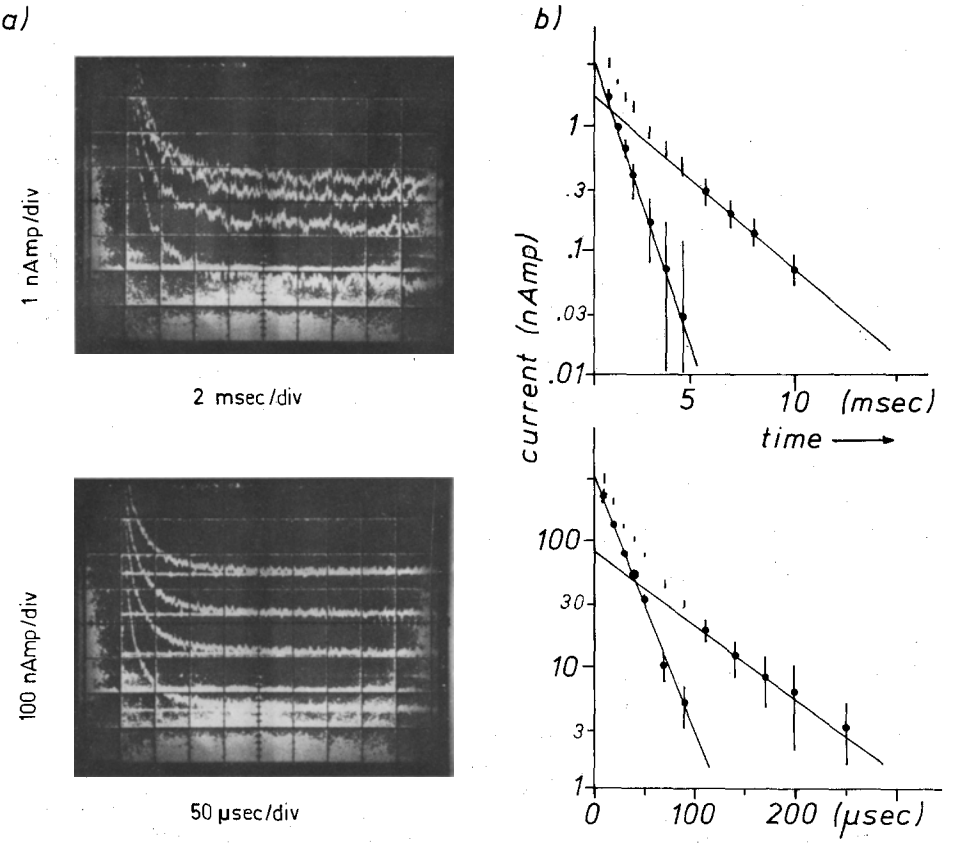

Fig. 3. (*a*) Raw current relaxation curves following a change of voltage from −150 mV to +150 mV (square wave with period of about 0.2 sec). Temperature = 33.7 °C. (*b*) Exponential analysis of the relaxation curves showing sequential subtraction of the identified components. *Upper:* longer times, yielding two components with $I_1 = 1.8 \pm 0.6$ nA, $\tau_1 = 3.3 \pm 0.6$ msec and $I_2 = 3.6 \pm 0.1$ nA, $\tau_2 = 0.9 \pm 0.2$ msec. *Lower:* shorter time scale, showing two further components with $I_3 = 85 \pm 15$ nA, $\tau_3 = 73 \pm 10$ μsec and $I_4 = 330 \pm 20$ nA, $\tau_4 = 21 \pm 4$ μsec. The means and standard deviations of the digitized curves are indicated: as each component is subtracted the cumulative uncertainty is given. Where no error bars are shown, the uncertainty limits correspond to the size of the symbols. Judging from the consistency of the points, uncertainties in the I's and τ's are considered generous (only the main curves are shown for the sake of clarity)

a priori, and for the moment the multi-exponential analysis may be considered simply as a *formal* description of the curves, without necessarily implying the existence of distinct processes. However, there is good evidence that several separate processes are involved, as will be seen in the Discussion section.

An example of both the raw curves and the subsequent exponential analysis is given in Fig. 3. As with all the results, several (usually four or

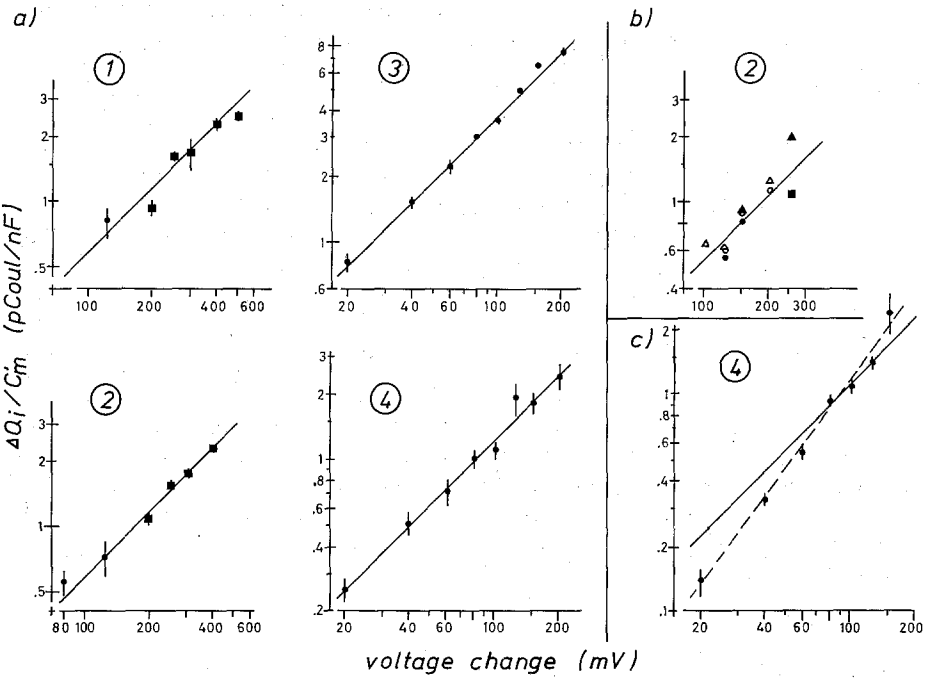

Fig. 4. Normalized displacement charge as a function of voltage for the four asymmetric relaxation components. Solid lines have slope = 1. Total voltage change across the membrane is given on the abscissa (e.g., for jump from −150 mV to +150 mV, V_{tot} = 300 mV). (*a*) Temp = 27.5 °C. For components **1** and **2** results for two different membranes formed during the same set of experiments are shown. (*b*) Scatter found among membranes formed on different days (uncertainty limits deleted for clarity). (*c*) Increased scatter often found with component **4**. A tendency toward a slope greater than 1 is also seen

more) curves were recorded in quick succession. The curves were then averaged, and the standard deviation taken as the experimental uncertainty. Curves of changes from $-V$ to $+V$ and from $+V$ to $-V$ were found to be accurate mirror images of each other.

The curves for each voltage setting were analyzed independently to eliminate bias in "choosing" the time constants. No correlation was found between the τ_i and the voltage jump. The results at a series of different voltages and with different membranes were then combined to calculate the corresponding relaxation amplitudes [Eq. (5)]. The voltage-dependence was checked graphically by plotting $I_o^i \cdot \tau_i / \mathbf{C}'_m$ versus V_m on double log paper (e.g., Fig. 4). All four relaxations were found to follow a linear relationship (slope = 1) with some small degree of scatter (arising mainly from the I_o^i's). No correlation was found between the (normalized) parameters and membrane area, showing that the effects were indeed related to

Table 1. Parameters of the four exponential components derived from the current-time curves for relaxation phenomena asymmetric with respect to voltage

Temp. (°C)	$\Delta C_1/C'_m$ [a] (%)	τ_1 [a] (msec)	$\Delta C_2/C'_m$ [a] (%)	τ_2 [a] (msec)	$\Delta C_3/C'_m$ [b] (%)	τ_3 [b] (μsec)	$\Delta C_4/C'_m$ [b] (%)	τ_4 [b] (μsec)
16	0.8(0.08)	3.5(0.9)	1.0(0.2)	0.67(0.06)	2.6(0.2)	170(11)	1.4(0.2)	17(2)
19.5	0.77(0.08)	3(1)	0.63(0.04)	0.60(0.05)	3.0(0.3)	160(20)	1.5(0.2)	18(3)
21.5	1.2(0.2)	3.4(0.8)	0.6(0.1)	0.7(0.1)	2.8(0.2)	150(30)	1.0(0.3)	17(3)
27.5	0.59(0.08)	2.9(0.6)	0.60(0.05)	0.6(0.1)	3.7(0.1)	140(12)	1.2(0.1)	18(3)
30.5	0.5(0.1)	2.7(1.4)	0.48(0.06)	0.65(0.15)	1.6(0.2)[a]	86(13)[a]	0.9(0.3)[a]	26(6)[a]
					3.9(0.2)	122(3)	1.3(0.1)	19(3)
33.7	1.1(0.1)	2.8(0.3)	0.46(0.12)	0.8(0.1)	1.0(0.1)[a]	66(10)[a]	1.0(0.2)[a]	21(3)[a]
					2.2(0.2)	63(8)	1.0(0.25)	17(2)

Numbers in brackets indicate the standard deviation of the results, considering the determinations at each voltage and for all membranes as separate trials. Examples of independent trials are given for components **3** and **4** at the two highest temperatures: the amplitude of component **3** shows significant variations while the other parameters are quite reproducible.

[a] Trials from 3/30/74.

[b] Trials from 3/22/74 unless otherwise noted.

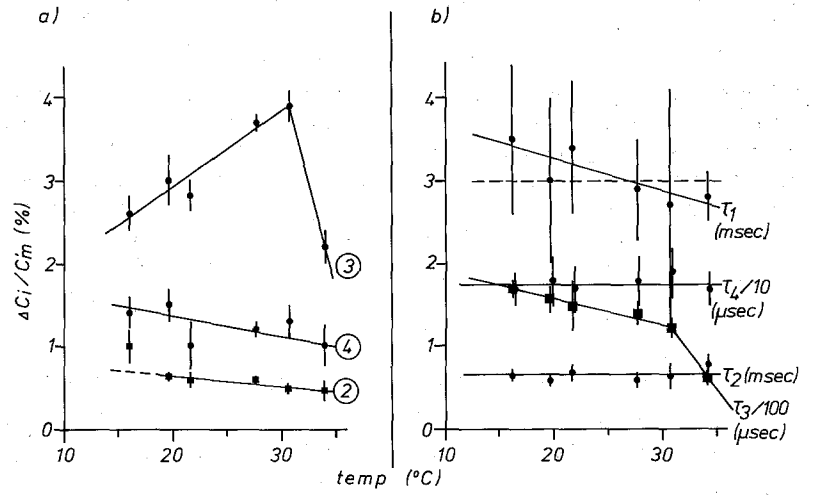

Fig. 5. Temperature-dependence of the asymmetrical relaxation components (error bars indicate standard deviation of the points). (*a*) Relaxation amplitudes (component **1**, the amplitude of which appears to vary rather erratically, is not shown): Components **2** and **4** show a slight trend to smaller values, while component **3** has a strong maximum at higher temperature (data from 3/22/74). (*b*) Time constants: Component **1** could be temperature-independent with the given experimental uncertainty, but a trend to shorter values at higher temperatures seems indicated. τ_2 and τ_4 show absolutely no temperature-dependence, while τ_3 shows a moderate slope at lower temperatures with a sharp drop at the high temperature end

the bilayer rather than the border region. The fastest relaxation showed the greatest scatter for each individual membrane, and there are indications that the amplitude is not wholly independent of voltage (*see* Fig. 4*c*).

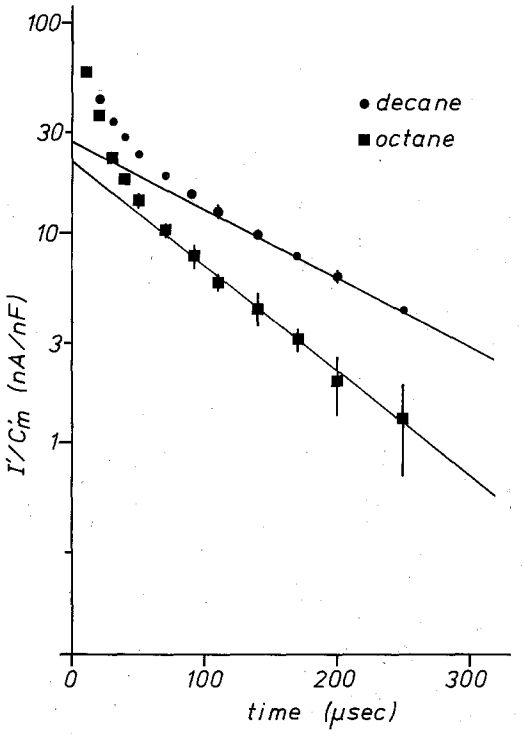

Fig. 6. Comparison of relaxation phenomena in membranes formed from oxidized cholesterol in decane and octane. Currents have been normalized with $\mathbf{C}'_m$. $\Delta V = 100$ mV. Exponential parameters are $I_3/\mathbf{C}'_m = 27 \pm 3$ nA/nF, $\tau_3 = 130 \pm 15$ μsec, $I_4/\mathbf{C}'_m = 51 \pm 6$ nA/nF, $\tau_4 = 21 \pm 4$ μsec for decane, and $I_3/\mathbf{C}'_m = 22 \pm 2$ nA/nF, $\tau_3 = 87 \pm 15$ μsec; $I_4/\mathbf{C}'_m = 86 \pm 13$ nA/nF, $\tau_4 = 12 + 4$ μsec for octane. Only the slower relaxation process is shown explicitly. Uncertainty limits correspond to the size of the symbols where not otherwise indicated

This might be caused by the presence of an even faster component that was not resolved at lower voltages.

The results of a temperature study are listed in Table 1 and shown graphically in Fig. 5. For the most part the slower components (**1** and **2**) were determined in different trials than the faster components (**3** and **4**). At the two highest temperatures all four components were also determined together. A major deviation between independent determinations is found only in the amplitudes of relaxation component **3**: when comparisons are to be made between component **3** and the other components, the amplitudes determined in the total surveys at 30.5 °C and 33.7 °C will be used. The characteristics of component **3** itself will be taken from the data of 3/22/74. The relative values of both determinations at 30.5 °C and 33.7 °C

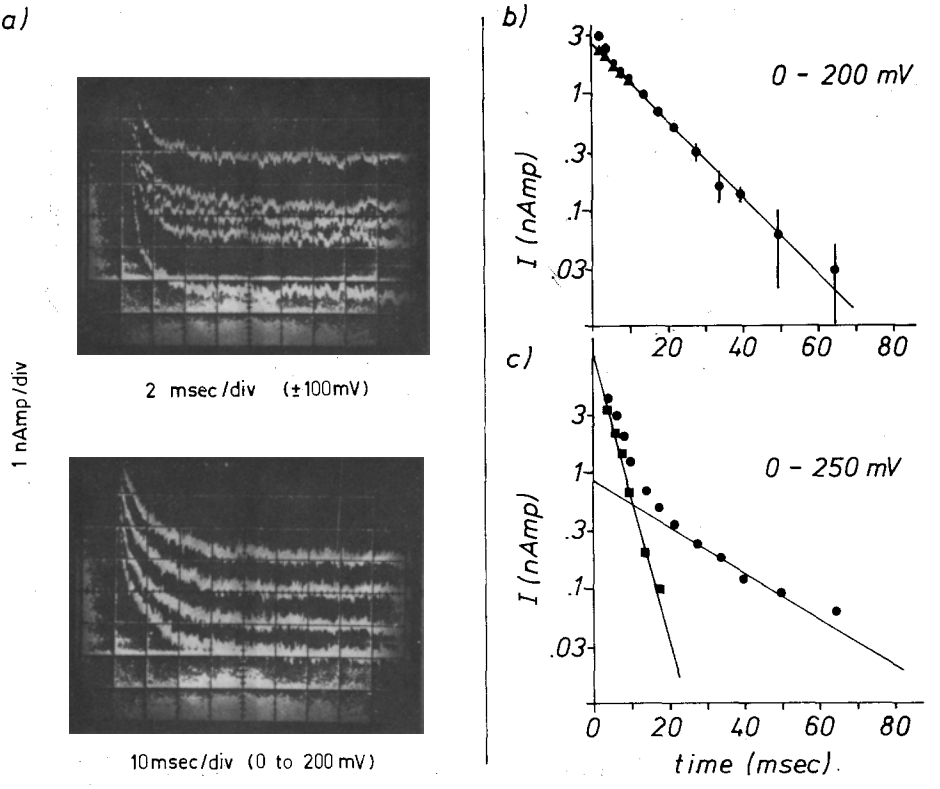

Fig. 7. Electrostrictive relaxation phenomena at $\mathbf{T}=16\,°C$. (*a*) Examples of raw data. (*b*) Relaxation analysis: (●) = points direct from 0 to +200 mV curve; (▲) effect of correction for $\Delta V = \pm 100$ mV (of significance only for $t < 8$ msec). The curve is well described by a single exponential ($I_1' = 2.7 \pm 0.2$ nA, $\tau_1' = 14 \pm 1$ msec). (*c*) Relaxation analysis for $\Delta V = 0$ to +250 mV, corrected for ±125 mV (only one trace could be made before bilayer broke): the curve requires a double exponential fit ($I_1' = 0.9 \pm 0.2$ nA, $\tau_1' = 42 \pm 6$ msec; $I_2' = 13 \pm 3$ nA, $\tau_2' = 7 \pm 2$ msec)

are consistent, and indicate a large decrease in amplitude of component **3** at the highest temperature.

The relaxation effects considered here will be of greatest interest and use if they can be shown to depend on the composition and structure of the membranes. That this is so is indicated in Fig. 6, where relaxation curves for oxidized cholesterol in decane and in octane are compared. The time constants are found to be significantly shorter when membranes were formed with octane ($\tau_3 = 87$ μsec, $\tau_4 = 12$ μsec) rather than with decane ($\tau_3 = 130$ μsec, $\tau_4 = 21$ μsec). In contrast to this, changing the salt solution to 1 M LiCl without buffer resulted in a slight lengthening of the time constants ($\tau_3 = 180$ μsec, $\tau_4 = 29$ μsec).

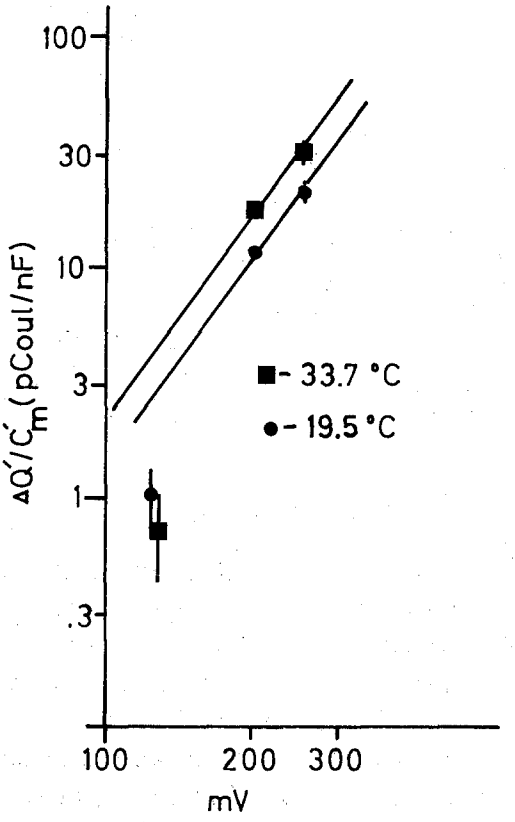

Fig. 8. Voltage-dependence of the symmetrical relaxation phenomena. The values at 250 mV are the sums of the two components found. Solid lines have a slope of 3, as is expected from Eq. (3)($\mathbf{Q}'_e \propto V^3$). Uncertainty limits correspond to the size of the symbols where not otherwise indicated

Symmetrical Effects

Relaxation currents for voltage jumps of zero to $+V$ volts were corrected for the asymmetric components, revealing any further relaxation processes[3] (Fig. 7). A change of the single exponential decay found at 200 mV into a complex decay at 250 mV (Fig. 7*b* and *c*) was observed in all cases. The total amplitudes at these voltages were found to follow the expected V^3 dependence, however, as seen in Fig. 8. The amplitude at 125 mV is almost an order of magnitude less than expected, and their time constants were much shorter than those found at the higher voltages. These results are summarized in Table 2.

3 A superscript "prime" (′) will be used to denote parameters of the symmetrical relaxation phenomena.

Table 2. Parameters of the exponential components derived from the current-time curves for relaxation phenomena symmetric with respect to voltage[a]

Temp.	$\Delta V = 125$ mV		$\Delta V = 200$ mV		$\Delta V = 250$ mV			
(°C)	$I' \cdot \tau'/\mathbf{C}'_m$ (pcoul/nF)	τ' (msec)	$I' \cdot \tau'/\mathbf{C}'_m$ (pcoul/nF)	τ' (msec)	$I'_1 \cdot \tau'_1/\mathbf{C}'_m$ (pcoul/nF)	τ'_1 (msec)	$I'_2 \cdot \tau'_2/\mathbf{C}'_m$ (pcoul/nF)	τ'_2 (msec)
16	0.4±0.1	2.3±1	16±0.8	14±1	16±4	42±4	39±8	7±1
19.5	1.0±0.3	4.8±1.5	12±1	16±3	10.4±1	42±6	10.4±1	7±1
21.5			9.2±0.6	22±2	11.0±0.6	17±3	9.5±0.6	3.4±0.5
	0.4±0.1	1.3±0.5	8±1	30±10				
27.5					9.0±0.8	10±1	9.0±0.8	2.3±0.4
33.7			18±1	20±3	16±1.5	14±3	14±2	3.8±0.5

[a] The indicated uncertainties are estimated from the graphs, as in Fig. 3. Each row represents a different bilayer.

Discussion

A question which must be settled at the outset is the possible contribution of various electrochemical processes to the relaxation phenomena observed. That electrode processes are not involved was shown in Fig. 2. Based on this experiment at minimal electrode area, one can conclude that diffusion polarization and double layer effects at the membrane surfaces are also probably negligible, as the known oxidation products of cholesterol (e.g., 7-dehydro, 7-keto and 7-hydroxycholesterol: McLaughlin, Szabo & Eisenman, 1971; Szabo, Eisenman, McLaughlin & Krasne, 1972; Lossen, Brennecke & Schubert, 1973) have no charged groups which could induce formation of diffuse double layers. In any case, the contribution of a dispersion due to diffuse double layer capacitance can be neglected above 100 Hz ($\tau < 3$ msec) (Coster & Smith, 1974).

The effect of the organic solvent (Fig. 6), which partitions strongly to the core of the BLM, also argues against surface reactions, as does the very minor effect of changes in the aqueous phase (e.g., 0.2 M NaCl + 8 mM phosphate *cf.* 1 M LiCl, no phosphate). Different impurities as well as different major components would be expected for the two aqueous compositions, so that the observed relaxations seem to be a property of the bilayer itself: the small changes observed could well reflect an influence of the aqueous phase on BLM structure (the BLM were mechanically and electrically less stable in 1 M LiCl than in 0.2 M NaCl).

The results with LiCl also make it highly unlikely that the observed relaxations might be caused by BLM conductive mechanisms. The background conductivity of oxidized cholesterol bilayers in 1 M LiCl is up to 100 times larger, and is considerably more noisy, than when the BLM

are formed in 0.2 M NaCl. That the relaxation results reported in this study show only minor differences in the two aqueous environments indicates that the relaxation phenomena are not due to the major conductive mechanism.

Asymmetric Relaxation Phenomena

Of the time constants listed in Table 1, only τ_3 shows a major temperature-dependence. That τ_3 can drop to about 40% of its highest value with no concomitant changes in either τ_2 or τ_4 is a good indication that component **3** represents a separate relaxation process, whether or not the multi-exponential analysis is valid for the total curve. The possibility remains that this separate relaxation process is superimposed on a continuum. Assuming the corresponding components of such a continuum to have the same characteristics as the faster and slower components, namely, a temperature-independent time constant, it is easy to show that the amplitude of the background can be only a small fraction of that of the separate component. As this represents a sharp dip in a hypothetical distribution curve in the range of time constant separating τ_2 and τ_4, components **2** and **4** also appear to represent distinct processes.

The relationship, if any, between the two slowest components cannot be determined with certainty from the data at hand: they may well represent a single, broad relaxation.

Other mechanisms having been excluded as described above, the asymmetric relaxation phenomena must result from molecular reorientations in the bilayer. The anticipated time scale of such motion can be taken from the Debye formula for the relaxation time constant of a dilute solution of noninteracting, spherical dipoles with radius r in a medium of viscosity η (e.g., Daniel, 1967):

$$\tau = (4\pi/k\mathbf{T})\,\eta r^3. \tag{6}$$

The main uncertainty in the evaluation of this expression is the value of the viscosity. Baessler, Beard and Labes (1970) used a value of 100 poise ("P") in an analysis of dipole relaxation times in liquid crystals formed from a mixture of cholesteryl chloride and cholesteryl myristate[4]. An independent estimate can be made from bilayer studies. Poste and Allison (1973) concluded that the viscosity of phospholipid bilayers is 100–1000 times that of water: the higher value may be taken for the more hindered hydrophilic head region of the bilayers. Further, the work of Szabo *et al.*

4 In the article by Baessler *et al.* the viscosity is printed as "100 cP", but the numerical results show that 100 Poise was used in the calculation.

(1972) indicates that bilayers of 7-dehydrocholesterol in decane have a viscosity about 30 times that of lecithin bilayers. Combining these results gives an expected viscosity of about 300 P. As a suitable value for r^3 we consider the molecular volume of cholesterol, $(4/3)\pi r^3 \simeq 10^{-21}$ cm^3 (Vandenheuvel, 1963). Substituting these values into Eq. (6) yields $\tau \simeq 20$ μsec. In addition, electrostatic interactions between the densely packed dipoles will lead to an increase in the time constant over that predicted by Eq. (6) (Daniel, 1967). Obviously, in spite of the uncertainty in η, we can expect molecular rotation with time constants in the range of 10^{-5} to 10^{-4} sec for oxidized cholesterol BLM. (An nmr study by Davis (1972) gave dipole correlation times of 1–5 μsec for lecithin BLM, consistent with these numbers.)

At this point it is of interest to note the fluorescence spectroscopy study of Yguerabide and Stryer (1971) on oxidized cholesterol bilayers. The authors found a considerable reduction in the anisotropy of the fluorescence parameters, from which they tentatively suggested that the rotational motion of their probe occurred in nanoseconds. The relevance of this to the rotational motion of the native constituents, especially the head group, must be questioned in the light of the present results. Certainly Eq. (6) would suggest that their figure is several orders of magnitude too fast. Many questions about depolarization mechanisms, probe location and disruptive effects of probes (e.g., Keith, Sharnoff & Cohn, 1973) remain: the advantage of the present study is that no probes are required.

The assignment of specific mechanisms to the three separate relaxation components seen is complicated by the complex composition of "oxidized cholesterol". Nevertheless, it seems unlikely that the various oxidation products would have relaxation parameters differing by orders of magnitude. Thus, the relaxation components identified in this study can, with some justification, be attributed to classes of relaxation mechanisms (motions) rather than classes of constituents.

I suggest that relaxation component **3** could result from a rotation of cholesterol about its long axis. This could explain the lack of saturation up to 250 mV (reduced component of the total dipole moment perpendicular to the long axis of the molecule). The temperature and amplitude-dependencies of component **3** (Fig. 5) are also consistent with such a mechanism: Arrhenius plots of both parameters yield free energies of activation of about 4 kcal/mole. Such a modest value could be expected from the small degree of asymmetry about the long axis of the molecule [according to Vandenheuvel (1963) the physical dimensions are roughly 6×8 Å].

The sharp decrease in τ_3 seen at 33.7 °C may reflect an increasing disorder or instability of some aspect of the system (the upper limit for stability of these BLM's is about 37 °C).

Components **1**+**2**, and **4** could arise from a reorientation of the dipole axis with respect to the perpendicular to the membrane surface. As a working hypothesis, I propose that component **4** may arise from the motion of individual dipoles while the much slower and broader tail of the asymmetric relaxation curve (components **1**+**2**) may be a cooperative realignment of groups of dipoles ("domains"). The temperature-dependence of the amplitudes of components **1**+**2**, and **4**, are similar in that a trend to smaller amplitudes at higher temperatures is found. The virtually flat τ versus **T** curves for all three components were also remarkable. These similarities are consistent with the hypothesis that the three components may be related, although they don't exactly form a continuum.

The concept of cooperative motion is well established in the liquid crystal field, and has also been invoked in bilayer studies (e.g., Hsu & Chan, 1973). The magnitude of the cooperative rotation required to account for the amplitude of components **1**+**2** is on the order of 0.2 degrees (*see* Appendix). With such small displacements the restoring forces could be expected to be in the elastic region, so that strict linearity of the **Q** versus V curves would be expected. Thus, this relaxation mechanism could explain both the time constant and the lack of saturation.

A similar phenomenon, involving cooperative alignment of domains of dipoles ("electrets") with time constants between 0.14 and 1.4 msec, has been proposed by Wobschall (1968) as a model of the nerve membrane. While such domains are entirely speculative, and there are many differences between oxidized cholesterol bilayers and nerve membranes, it is noteworthy that dielectric relaxation phenomena with suitable time constants can be observed in lipid bilayers.

Symmetric Relaxation Phenomena

The data presented in Table 2 are difficult to classify simply. Although the individual relaxation curves were unambiguous (e.g., Fig. 7 for $\mathbf{T}=16$ °C), there is more scatter than for the asymmetric effects. White (1970*b*) also found that the deviation between membranes was greater than the experimental error limits, and concluded that it represented variations in the membranes themselves. In spite of these difficulties it is possible to identify certain trends in the data. The strictly single-exponential character of the relaxation at 200 mV contrasts sharply with the more

complex time course at 250 mV, so that there appears to be a voltage-dependence to the temporal response of the electrostrictive phenomena. This conclusion is supported by the much shorter time constants found at 125 mV. The effects at 250 mV may to some extent be "pathological", as the bilayers usually lasted only a few seconds at this voltage.

With the exception of τ'(200 mV), the time constants show a trend to lower values at higher temperatures, which could be indicative of lower membrane viscosities. No trends in relaxation amplitude with temperature can be distinguished.

The voltage-dependence of the amplitudes follows the predicted R.A.$\propto V^3$ behavior in the rather restricted range of 200–250 mV. The amplitudes of the relaxations detected at 125 mV are an order of magnitude lower than would fit the values at the higher voltages. (An extension of the relaxation studies to still lower voltages is not feasible due to the large "background" from the asymmetric relaxations.) The apparent discrepancy may be related to the voltage-dependence of the time constants: further symmetric relaxations are presumably present at much longer time constants (e.g., Andrews *et al.*, 1970; Wobschall, 1972). The corresponding currents would be below the resolution of the present apparatus and would best be studied with a-c techniques. Even at 200 and 250 mV the values of Table 2 differ from White's results (1970*b*) and it thus seems likely that further relaxation components remain unresolved at the higher as well as at the lower voltages.

Further analysis is not possible at the moment, except to note that BLM thinning by transfer of solvent to microlenses or the border could rationally account for at least some of the electrostrictive relaxations found. Assuming a diffusion constant for decane in the hydrocarbon interior of about 10^{-6} cm^2/sec, and an inter-microlens distance of 10 μ (e.g., White & Thompson, 1973), time constants of about 40 msec can be expected (*see*, e.g., White, 1970*b*). The much faster change seen at $V=$ 125 mV will presumably be caused by some other process, such as a reorganization of the BLM constituents throughout the thickness of the membrane.

A more detailed study of the electrostrictive phenomena will be reported in a future publication.

General

The technique described in this paper complements the many methods presently used in the study of bilayer structure and dynamics. As it makes

use of properties of the native bilayer constituents, uncertainties about probe artifacts are abolished. The major disadvantage is that a definite identification of the observed relaxations with particular processes in the bilayers is not always obvious. This is an area which should yield to future investigations, however, and in the meantime the method should be useful for comparative studies. Examples arising from the present study are:

(a) the shorter time constants found with octane demonstrate once again the importance of the solvent in planar "bilayers" (Haydon, 1970).

(b) while the specific capacitance of the bilayer increases by over 10% at the higher voltages the asymmetric relaxations remain unaffected (no voltage-dependence was found). The mechanical distortion of the membrane apparently does not influence the mobility of the reorienting groups significantly, perhaps because of the relative sizes of the head and tail regions of cholesterol.

(c) it has often been suggested that dipole reorientation might be the cause of membrane breakage at higher voltages (e.g., Henn & Thompson, 1969). I have found that oxidized cholesterol BLM usually break within seconds when pulsed between 0 and 250 mV but are more stable when a symmetrical ± 250 mV square wave is applied. The latter treatment will cause double the dipole realignment, while keeping the mechanical forces constant. For this membrane, the electromechanical forces (shear or deformation) are obviously of much greater importance in membrane breakage than dipole reorientations.

Previously, capacitance measurements have been used to determine average structural properties of BLM (e.g., Hanai *et al.*, 1965*a*; Coster & Smith, 1974), or to study surface adsorption phenomena (e.g., Rosen & Sutton, 1968; Wobschall & Ohki, 1973). The present work shows that high resolution frequency-dependence studies are a potential source of information about BLM dynamics as well.

That many authors have failed to see frequency-dependent effects of the type I have described must be attributed to insufficient resolution (e.g., Hanai *et al.*, 1965*a*, were limited to about 1% accuracy). The advantage of the pulse technique is its speed: problems of drift and mechanical instability are reduced. Although the automated procedure introduced by Coster and Smith (1974) greatly alleviates such problems, the potentially higher resolution of a-c methods may not be realizable under many conditions.

Both dipole relaxation phenomena and electrostrictive effects are to be expected in most types of BLM. I have found asymmetric relaxations in dioleoyllecithin BLM (Sargent, 1975*b*), and Bamberg and Läuger (1973)

have reported an electrostrictive effect ($\tau = 3$ msec) with the same substance. Thus such phenomena are also amenable to study in membranes much more "fluid" in character than oxidized cholesterol BLM are.

There are many potential areas of application for this technique. The detection of phase transitions, the effect of membrane active materials on membrane structure (especially when conductivity mechanisms are not involved, such as many hormone-receptor or antibody interactions), the detection of the binding of molecules to BLM (e.g., Vasquez, Parisi & de Robertis, 1971), the different structural and dynamic properties of solvent-free bilayers (Montal & Mueller, 1972), and the effects of BLM stabilizing procedures (e.g., King & Steinrauf, 1972) are but a few examples.

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Appendix

Effect of Cooperative Dipole Rotation on Bilayer Capacitance

The potential energy of the dipoles in the membrane is given by

$$\begin{aligned} PE^{\text{dip}}(\text{per unit area}) &= n \cdot \mathbf{u} \cdot \mathbf{E} \\ &= n \cdot u \cdot E \cdot \cos\theta \end{aligned} \tag{A.1}$$

where n = no. of dipoles/cm^2 = 2.5×10^{14}/cm^2 (i.e., 40 Å^2 per cholesterol molecule), $\mathbf{u}$ = dipole moment of cholesterol $\simeq 3$ DU $\simeq 10^{-24}$ coul cm, $\mathbf{E}$ = field strength (V/cm) and θ = angle between $\mathbf{u}$ and $\mathbf{E}$.

The potential energy stored in a capacitor is

$$PE^{\text{cap}}(\text{per unit area}) = 1/2\ CV^2. \tag{A.2}$$

If the dipoles realign in the applied electric field, thereby changing the capacitance and reducing the field strength in the bilayer, we may apply Eqs. (A.1) and (A.2), giving $\Delta PE^{\text{cap}} = \Delta PE^{\text{dip}}$, or explicitly,

$$1/2\ V^2 \cdot \Delta C = n \cdot u \cdot E(\cos\theta_f - \cos\theta_i) \tag{A.3}$$

where θ_i and θ_f are the initial and final directions of the dipole moments with respect to the field. For $\Delta C/C = 1\,\%$, with $C = 0.4\ \mu\text{F/cm}^2$, $V = 100$ mV, and membrane thickness = 40 Å, Eq. (A.3) becomes $(\cos\theta_f - \cos\theta_i) = 6 \times 10^{-6}$. If $\theta_i \simeq 0°$, then $\theta_f = 0.2$ degrees.

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