Biochimica et Biophysica Acta, 509 (1978) 9—20

BBA 77999

OXIDIZED CHOLESTEROL BILAYERS

DEPENDENCE OF ELECTRICAL PROPERTIES ON DEGREE OF OXIDATION AND AGING

RUSH L. ROBINSON and ALFRED STRICKHOLM

Physiology Section, Medical Sciences Program, Indiana University School of Medicine, Bloomington, Ind. 47401 (U.S.A.)

(Received December 15th, 1977)

Summary

Black lipid membranes made from oxidized cholesterol were examined for their specific resistance, capacitance, and physical stability, as a function of cholesterol oxidation time and of age. Membranes formed from cholesterol oxidized in *n*-octane were not physically stable even after 7 h of oxidation unless they were aged for at least a month. Membranes formed from cholesterol oxidized in decane and tetradecane (1 : 1) were stable immediately after 2—6 h of oxidation. Oxidation times outside this range produced unstable membranes. After 1 month storage, membranes from cholesterol solutions oxidized in decane and tetradecane from 0.75—3 h were stable. After 11 months, only the 0.75 oxidation time produced stable membranes. Storage in nitrogen retarded the aging process. After initial forming of the membrane, total membrane area and capacity increased and then stabilized, although specific capacity and resistance did not change, indicating inherent stability in the bilayer's intrinsic electrical properties. Bilayers formed soon after cholesterol oxidation had membrane capacity which ranged from 0.42 to 0.55 $\mu F/cm^2$. Specific membrane resistance ranged initially from $2 \cdot 10^6$ to $37 \cdot 10^6$ Ω/cm^2 in 0.2 M NaCl with lower resistances in the more oxidized membranes. With aging, membrane capacity decreased gradually over 11 months to values approaching 0.1 $\mu F/cm^2$ indicating membrane thickening. Membrane resistance ordinarily decreases with storage time. The rate of these changes with age is dependent on the extent of initial cholesterol oxidation and subsequent oxidation, with long term stability best in the least oxidized membranes.

Introduction

In aqueous media appropriate lipids spontaneously thin down to the dimensions of a cell membrane and form a black lipid bilayer or membrane. With

appropriately adsorbed proteins, black lipid membranes have been used to reproduce biologically significant phenomena such as active transport, ion selectivity, antigen-antibody reactions, excitability, and photo-electric effects [1,2].

Black lipid membranes separating two aqueous phases have been formed from a variety of amphipathic lipids including oxidized cholesterol [3]. Although oxidized cholesterol is widely used in membrane-forming mixtures, its physical properties and composition have not been completely established. It is known, however, that 7-hydroxycholesterol and 7-ketocholesterol are principle components [4].

Electrically, a lipid membrane can be represented as a resistor in parallel with a capacitor [5], where the electrolyte solutions represent the conducting plates of the capacitor and the hydrocarbon membrane the separating dielectric [6]. Changes in the physical properties of the black lipid membrane are usually reflected as changes in resistance or capacitance [3]. This is of use for studying the interaction of components incorporated into the black lipid membrane such as ion transport proteins [7]. Because electrical parameters reveal much about lipid membranes, it is important to know what factors effect them. It has been shown that both resistance and capacitance are dependent on the type of alkane solvent and lipid used in forming membranes. Some types of membranes are also effected by the composition of the surrounding electrolyte media; such as its ionic constituents, ionic strength and pH [8]. An electric field applied across the membrane can also alter membrane capacitance [9,10,6]. White [6] found that the capacitance was effected by the age of the lipid solution, the ambient temperature and the duration of the experiment.

The purpose of this investigation was to delineate the factors of oxidized cholesterol solutions which determine the physical features of formed black lipid membranes such as stability, capacitance, resistance, thickness, etc.

Methods

Cleaning procedures and oxidation of cholesterol

Black lipid membranes are sensitive to contamination, especially by surface-active agents. Thus, the water used for cleaning and buffers was double distilled; first with a tin lined still (Barnstead) and then in glass with potassium permanganate. Glassware was cleaned in a dichromate/H_2SO_4 bath, and rinsed several times with water.

The plastic chambers were soaked 24 h in 3 M NaOH followed by a rinse in weak HCl to neutralize the NaOH, then rinsed several times with water. The teflon chambers which supported the membrane were soaked 24 h in 3 M NaOH, boiled for 45 min in 3 M NaOH, rinsed with water, soaked for 1 h in 0.1 M HCl in ethanol to neutralize the NaOH, rinsed again with water, boiled for 10 min in ethanol, soaked for 1 h in fresh ethanol, and then oven dried. Teflon tubing was also used as a support for some of the membranes. It was used only once so extensive cleaning was unnecessary. It was boiled in ethanol, soaked overnight in water, and then oven dried. The glass disposable pipettes used to form the membranes were soaked for at least 48 h in chloroform/methanol (1 : 1, v/v), then boiled for 20 min in chloroform/methanol, and then oven dried.

The black lipid membrane-forming solutions were prepared similar to the method used by Tien et al. [11]. A solution of 4% (w/v) cholesterol in an alkane solvent was refluxed in the presence of molecular oxygen. Several sets of solutions were prepared. The first set was prepared with 4 g of cholesterol (Matheson, Coleman, and Bell, reagent grade) in 100 ml of *n*-octane (Eastman, practical grade). The mixture was stirred and refluxed at 126°C, while molecular oxygen was bubbled through it at the rate of 2—3 bubbles/s. 10-ml samples were removed at various intervals up to 7 h. The samples were stored in teflon-capped glass tubes. The actual membrane-forming solution was prepared by diluting the oxidized cholesterol/*n*-octane sample (1 : 1, v/v) with a 1 : 1 (v/v) mixture of decane (Eastman, practical grade) and tetradecane (Eastman, practical grade). If the oxidized cholesterol/*n*-octane sample had a white precipitate, it was redissolved by heating before the sample was mixed with the decane/tetradecane.

All the other solutions were prepared and stored in a similar manner to the solutions above except the *n*-octane was replaced with a 1 : 1 (v/v) mixture of decane/tetradecane, and it was unnecessary to dilute the samples to make black lipid membranes. Because of the decane/tetradecane, the solutions refluxed at 198°C.

The second set of samples had each sample prepared individually from 1.2 g cholesterol in 30 ml of decane/tetradecane (1 : 1, v/v). Each sample was refluxed for different lengths of time up to 4 h, and then stored at room temperature for approx. 24 h before it was used to make membranes.

The third set of solutions were prepared from 5 g of cholesterol in 125 ml of decane/tetradecane, and 10-ml samples were taken at intervals up to 6 h. Following its removal from the reaction mixture each sample had molecular nitrogen bubbled into it. The samples were then stored for 2 months at room temperature before they were used to form membranes.

The final set of solutions were prepared from 5 g cholesterol in 125 ml of decane/tetradecane. 10-ml samples were taken at various intervals up to 8 h. They were kept at room temperature and used to form membranes after storage of 1, 4, and 11 months in glass vials.

The electrolyte solution surrounding the black lipid membranes was 0.2 M NaCl with 0.02 M sodium phosphate buffer (pH 7.0 prepared from reagent grade chemicals and double distilled water).

Membrane apparatus

The membranes were formed on two different type teflon supports. One support was formed from a Teflon hollow bar containing a small hole (1—2 mm diameter) over which the membrane was formed. This open hole was submerged in the buffer which filled a plastic chamber having a front of 1.5 mm clear plastic. It was visualized with a microscope having a 10× eyepiece with calibrated graticule and a 5× Ultropak (Ernst Leitz Wetzlar) objective which has an epi-illuminating light source. The Ultropak epi-illuminator simplified visualization of membrane interference fringes during thinning and eliminated the difficult optical alignment problems present with a separate light source and telescope viewing optics. A nylon valve with syringe in the upper open end of the teflon chamber was used to draw buffer through the membrane forming

hole into the chamber. When the valve was closed, the volume and pressure on one side of the formed membrane was thus held constant. Also in the teflon chamber was attached a polyethylene tubing salt bridge containing 3 M KCl and 5% non-ionic agar (Bausch and Lomb, Agarose). A similar salt bridge was connected to the plastic chamber. The two agar/salt bridges connected to saturated KCl/calomel cells. The other method utilized teflon tubing in place of the teflon bar and, since it was only used once, simplified the cleaning procedures.

Electrical circuitry and data analysis

The electrical circuit (Fig. 1) used to measure the electrical properties of the membranes is similar to that used by Jain et al. [7]. An oscillator with push putton selection (Kron-Hite, model 440A) was used to provide voltage input and precise frequency reproducibility. The electrometer operational amplifier (Analog Devices, model 310J) had an input impedance of $3 \cdot 10^{11}$ Ω and an open loop gain of 10^5. The feedback resistors (R_f) for current measurement ranged from 10^9 to 10^5 Ω and were relatively calibrated to 0.1%. The resistor selecting switch was of the shorting type to prevent high voltage transients during switching which often broke the membrane. The entire apparatus was located in a grounded Faraday cage.

From Fig. 1 the output potential (V_o) is:

$$V_o = V_- - IR_f = -A V_- \tag{1}$$

Thus:

$$V_- = IR_f/(1 + A) \tag{2}$$

Since $IR_f \leqslant 10$ V and $A = 10^5$, $V_- \leqslant 10^{-4}$ V, and: $V_o = -IR_f$. (3)

V_- is therefore a virtual ground, and the potential across the membrane is V_{in}.

The preceding equations describe the solid junction circuit of Fig. 1. With the apparatus used here, the operational amplifier was connected to the membrane through the series resistances of electrolytes, the buffer, the 3 M KCl/salt bridges, and the calomel cells. The calomel cell potentials are, however, equal and opposite here and thus cancel. In addition, the sum of these series

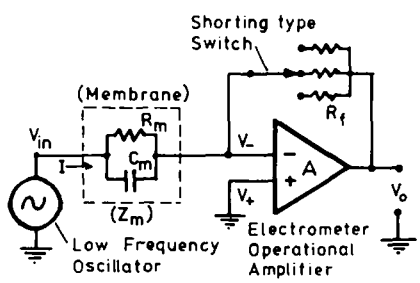

Fig. 1. Equivalent circuit for measuring membrane impedance, where: V_{in} is input voltage, I is current through the membrane, R_m is membrane resistance, C_m is membrane capacitance, Z_m is membrane impedance, R_f is feedback resistance, V_o is output of operational amplifier, A is gain of operational amplifier.

resistances were about $10^4\ \Omega$. This was negligible compared to the membrane impedance, Z_m, which was usually around $10^8\ \Omega$ in 0.2 M NaCl. Thus, only the membrane potential (V_{in}) and membrane impedance (Z) need be considered in the circuit analysis here.

The membrane current I is: $I = V_{in}/Z_m$ (4)

Substituting Eq. 4 into Eq. 3:

$$V_o = -(R_f/Z_m)V_{in} \quad (5)$$

The fact that a black lipid membrane can electrically be represented by a parallel resistor-capacitor circuit (Fig. 1) was used to analyze the data. The impedance of a parallel resistor-capacitor circuit, utilizing complex notation for sine wave analysis, is:

$$1/Z_m = 1/X_c + 1/R_m \quad (6)$$

where: Z_m is the total membrane impedance, X_c is $1/j\omega C_m$, the impedance due to the membrane's capacitance; where j is the square root of -1, and $\omega = 2\pi f$, where f is the frequency (cycles/s), R_m = the membrane resistance. Substituting Z_m from Eqn. 6 into Eqn. 5 gives:

$$V_o/R_f = -V_{in}(j\omega C_m + 1/R_m) \quad (7)$$

V_o/R_f is thus a complex number with real and imaginary parts, and has an absolute magnitude equal to:

$$|V_o/R_f| = |V_{in}|[(\omega C_m)^2 + (1/R_m)^2]^{1/2} \quad (8)$$

Rearranging the terms and squaring:

$$(|V_o|/|V_{in}|)^2 \cdot (1/R_f^2) = \omega^2 C^2{}_m + 1/R_m^2 . \quad (9)$$

Eqn. 9 is in the form of a binomial equation, $y = ax + b$. If $(|V_o|/|V_{in}|)^2 \cdot (1/R_f^2)$ (y axis) is plotted against ω^2 (x axis), then C_m^2 will be the slope and $1/R_m^2$ the y-intercept (see Fig. 2).

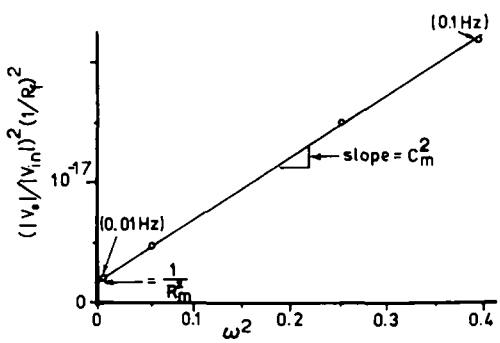

Fig. 2. Plot of data for determining membrane capacitance and resistance. 1.5 h oxidized solution measured after 24 h aging. Total membrane capacity and resistance are $7.07 \cdot 10^{-9}$ μF and $7.45 \cdot 10^8\ \Omega$, for a membrane area of $1.36 \cdot 10^{-2}$ cm².

While the preceding method was excellent for determining membrane capacitance from the slope, the y-intercept of a least square fit of data points was not satisfactory for determining R_m. Membrane resistance was best determined from d.c. measurements or by Eqn. 11 below using the membrane capacitance derived from a least squares fit of data plotted according to Eqn. 9, and $|V_{in}|$ and $|V_o|$ of the lowest frequency used.

If Eqn. 6 is solved for Z_m we obtain:

$$|Z_m| = [R_m^2/(1 + \omega^2 C_m^2 R_m^2)]^{1/2} . \tag{10}$$

Solving for R_m we have:

$$R_m = [|Z_m|^2/(1 - \omega^2 C_m^2 |Z_m|^2)]^{1/2} \tag{11}$$

At a given frequency $|Z_m|$ is determined from Eqn. 5 as $R_f|V_{in}|/|V_o|$, and C_m is derived from the slope of the data points of Eqn. 9. Eqn. 11 thus gives the membrane resistance (R_m) for a given ω.

The membrane capacitance (C_m) ideally is:

$$C_m = \epsilon A/4\pi t . \tag{12}$$

Where: A is the membrane surface area, ϵ is the membrane dielectric constant, and t is the membrane thickness. Capacitance thus monitors changes in the membrane surface area and/or thickness in addition to changes in dielectric constant. Ordinarily the dielectric constant is unlikely to vary over the course of an experiment [6]. From measuring membrane surface area, the specific membrane capacitance is obtained as: $C_m/A = \epsilon/4\pi t$, which thus can monitor changes in membrane dielectric or thickness [6].

Membrane experiments

Prior to forming a black lipid membrane, the feedback resistance (R_f) was set at $1 \cdot 10^5\ \Omega$ and a small d.c. potential, 1—2 mV, was applied across the circuit. The voltage output (V_o) was recorded with a pen recorder (Hitachi Perkin-Elmer, model 165). Bubbles of the oxidized cholesterol solution were blown with a disposable pipette past the small hole in the Teflon support until one of the bubbles would adhere and form a membrane. When this happened the voltage output would dro close to zero, since the membrane impedance (Z_m), usually around $10^8\ \Omega$, far exceeded the feedback resistance (R_f) (see Eqn. 5). The membrane was optically examined and if there were attached bubbles, it was rejected. Otherwise, it was observed until the region within the Plateau-Gibbs border ceased to show colored interference fringes and turned dark gray. This indicated that the membrane had completed thinning [3], and the time required was called thinning time. The membrane was left for 10 min with a small d.c. potential across it to test its stability and measure the membrane resistance. If the membrane did not break within the first 10 min after thinning, it was usually stable for the duration of the experiment. A series of sine waves of 20—45 mV and from 0.01 to 0.5 cycles/s was then generated across the membrane. Measurements were made at 3—10 different frequencies, more typically four. A measurement at each frequency ordinarily consisted

of a minimum of eight cycles to allow for settling of the initial turning on transients. Just preceding the series of sine waves the membrane diameter to the Plateau-Gibbs border was measured. Following the sine waves the membrane sat for another 10 min with no voltage across it. Then the same series of sine waves was repeated. In early experiments the membrane diameter was measured only once just preceding the first series of sine waves. In later experiments it was measured just preceding each series. All the experiments were performed at room temperature, and the membrane would ordinarily last for the experimental measuring time of 35 min.

Results

(1) Solutions prepared with n-octane

The first set of samples used cholesterol oxidized in *n*-octane and diluted 1 : 1 (v/v) with decane/tetradecane. All the samples, from 0.5 to 7 h oxidation, were clear and colorless with a white precipitate. Several days after preparation, membrane solutions were made from these samples, but they failed to form membranes. Only after 2 weeks of aging would these samples form membranes. The membranes had white crystals, probably of unoxidized cholesterol, in their Plateau-Gibbs borders and were unstable. They broke shortly after thinning probably due to the crystals. Other authors have reported similar findings [11]. It required more than a month of aging for even the more oxidized solutions to form stable membranes.

(2) Solutions prepared with decane/tetradecane

Three different sets of solutions were prepared with cholesterol oxidized in decane/tetradecane (see Methods). In the first set, each sample was prepared individually and then tested within 24 h after preparation (Table I). A second

TABLE I

EFFECT OF OXIDATION TIME ON MEMBRANE PROPERTIES

4% Cholesterol oxidized in decane/tetradecane (1 : 1, v/v) and stored in air-sealed vials for 24 h. — indicates broke before measurement. Subscripts 1 and 2 refer to measurements taken at 10 and at 20–30 min after membrane formation.

Oxidation time (h)	Membrane capacitance ($\mu F/cm^2$)	Membrane resistance ($10^6\ \Omega/cm^2$)	Membrane surface area ($1 \cdot 10^{-2}\ cm^2$)
1.0	C_{m_1} 0.49	R_{m_1} 20.1	1.58
	C_{m_2} —	R_{m_2} —	—
1.25	C_{m_1} 0.49	R_{m_1} 27.0	1.74
	C_{m_2} 0.50	R_{m_2} 26.7	1.80
1.5	C_{m_1} 0.52	R_{m_1} 10.1	1.37
	C_{m_2} 0.55	R_{m_2} 9.97	1.32
2.0	C_{m_1} 0.52	R_{m_1} 37.3	2.09
	C_{m_2} 0.52	R_{m_2} 36.7	2.09
4.0	C_{m_1} 0.46	R_{m_1} 1.93	1.42
	C_{m_2} 0.42	R_{m_2} 1.95	1.42

TABLE II

EFFECT OF STORAGE IN NITROGEN

4% cholesterol oxidized in decane/tetradecane (1 : 1, v/v) and stored under nitrogen for 2 months. — indicates membranes will not form.

Oxidation time (h)	Membrane capacitance ($\mu F/cm^2$)	Membrane resistance ($10^6\ \Omega/cm^2$)	Membrane surface area ($1 \cdot 10^{-2}\ cm^2$)
0.5	—	—	—
1.0	C_{m_1} 0.54	R_{m_1} 7.52	1.37
	C_{m_2} 0.54	R_{m_2} 8.48	1.37
1.5	C_{m_1} 0.52	R_{m_1} 24.4	2.35
	C_{m_2} 0.53	R_{m_2} 28.4	2.48
2.0	C_{m_1} 0.56	R_{m_1} 20.6	2.22
	C_{m_2} 0.59	R_{m_2} 23.9	2.41
2.5	C_{m_1} 0.51	R_{m_1} 18.4	1.74
	C_{m_2} 0.51	R_{m_2} 15.9	1.68
3.0	C_{m_1} 0.54	R_{m_1} 25.6	2.03
	C_{m_2} 0.53	R_{m_2} 28.4	2.09
4.0	C_{m_1} 0.51	R_{m_1} 20.8	2.17
	C_{m_2} 0.53	R_{m_2} 17.3	2.20
5.0	C_{m_1} 0.53	R_{m_1} 13.3	2.09
	C_{m_2} 0.54	R_{m_2} 15.0	2.22
6.0	C_{m_1} 0.45	R_{m_1} 7.62	2.41
	C_{m_2} 0.46	R_{m_2} 7.64	2.55

set was prepared and stored in vials with nitrogen at room temperature for 2 months before testing (Table II). The third set was stored in sealed vials with air at room temperature and tested at intervals of 1, 4, and 11 months (Table III).

With all the above three sets, the samples which were refluxed for only a short period of time were clear and colorless and contained a white precipitate apparently of cholesterol. They generally produced membranes with white crystals in their Plateau-Gibbs borders, had thinning times less than 30 s, and were unstable. In Table III the 0.25 and 0.75 h oxidation time sample and in Table I the 1.0 and 1.25 h oxidation time sample behaved this way. Usually solutions which were refluxed longer than 1.5 h had no precipitate. As oxidation continued, the solutions became progressively more yellow. This observation agrees with previous reports on the oxidation of cholesterol with molecular oxygen [12].

The well oxidized solutions which were stored without nitrogen deteriorated within a month (Table III). Solutions oxidized longer than 3 h produced unstable membranes. These membranes had sharp clear interference patterns and very long thinning times, usually greater than 3 min. Often they broke spontaneously before thinning was complete. In contrast to air storage (Table III), storage in nitrogen (Table II) preserved the membrane-forming properties of the solutions. Solutions refluxed for up to 6 h and stored for 2 months in nitrogen still produced stable membranes.

TABLE III

EFFECT OF AGING OF OXIDIZED CHOLESTEROL ON MEMBRANE PROPERTIES

4% cholesterol oxidized in decane/tetradecane (1 : 1, v/v) and stored in air-sealed vials for 1, 4, and 11 months. — indicates membranes will not form.

Oxidation time (h)	1 month		4 months		11 months	
	Membrane capacitance ($\mu F/cm^2$)	Membrane resistance ($10^6\ \Omega/cm^2$)	Membrane capacitance ($\mu F/cm^2$)	Membrane resistance ($10^6\ \Omega/cm^2$)	Membrane capacitance ($\mu F/cm^2$)	Membrane resistance ($10^6\ \Omega/cm^2$)
0.25	—	—	0.40	12.1	0.22	23.6
0.75	0.64	14.2	0.32	12.9	0.18	24.0
1.5	0.45	7.65	0.28	10.3	—	—
2.0	0.43	2.65	0.26	2.28	0.11	0.26
2.5	0.44	2.43	0.29	1.03	—	—
3.0	0.49	0.51	0.12	0.39	—	—
4.0	—	—	—	—	—	—

(3) Electrical properties of membranes formed from solutions prepared with decane/tetradecane

In Tables I and II, C_{m_1} is the specific capacitance measured about 10 min after membrane thinning, C_{m_2} is the specific capacitance measured later (approx. 20—30 min after thinning), and R_{m_1} and R_{m_2} are the corresponding specific resistances. In Table III, C_m and R_m were determined 10 min after membrane thinning. The apparent constancy of specific membrane resistance and capacity with changes in membrane area indicate that the intrinsic membrane properties are stable once the membrane is formed.

Tables I and II show that in most cases the thinned area expands between the first and second series of measurements. Since the membrane is formed over a hole of fixed size, the expansion must come at the expense of the Plateau-Gibbs border. Those membranes which did not change in size had minimal Plateau-Gibbs borders and probably had reached equilibrium early.

(A) Membranes formed from solutions aged for approx. 24 h (Table I). The lightly oxidized 1- and 1.25-h solutions produced membranes with typical capacitances [3] and high specific resistances. Both these membranes had crystals in their Plateau-Gibbs borders while the more oxidized solutions did not. The 1 h membrane was unstable and did not last long enough to allow a second electrical measurement. The 1.5- and 2-h solutions produced membranes with specific capacitances and resistances similar to be 1 h oxidation time but had greater physical stability. The 4-h oxidized solutions produced a membrane with a lower specific capacitance and lowered specific resistance. More than 4 h of oxidation produced unstable membranes. If the 4 h oxidation solution of Table I is compared with that of Table II, it can be seen that within 24 h after preparation, heavily oxidized solutions begin to change unless stored in nitrogen.

(B) The effects of storage in nitrogen. Table II shows the specific capacitances and resistances for the solutions stored in nitrogen for 2 months. Except for the lightly oxidized 0.5 h solution, all the solutions formed membranes without crystals in their Plateau-Gibbs borders. These membranes with crystals broke shortly after thinning. All the membranes except the one formed from the heavily oxidized 6 h solution had specific capacitances close to the value (0.57 μF/cm^2) measured by Tien and Diana [3]. Specific resistance (Table II) seemingly increases, then decreases with the degree of oxidation in a manner similar to that produced by aging (Table III). Nitrogen storage thus not only preserved the membrane-forming qualities of the solutions but also the initial values for specific capacitance and specific resistance.

(C) The effects of aging on the specific capacitance and specific resistance (Table III). After 11 months of sealed storage in air, only the solutions which were initially lightly oxidized produced stable membranes. Table III shows that after 1 month of aging all the solutions except the 0.75 oxidation time solution had specific capacitances below the initial value around 0.5 μF/cm^2 (Table I). After 4 months the specific capacitances of most solutions fell by approximately one half, and after 11 months to approximately a quarter of their 1-month values. The specific resistance values (Tables I—III) measured here in 0.2 M NaCl are near that reported by others (10^8 Ω/cm^2 in 0.1 M solutions [3,1]). The effects of solution aging on specific resistance was different from

its effects on specific capacitance. With aging, specific resistance ordinarily declines, but for light oxidation it seems to initially increase (Table III).

Discussion

The data indicates that the degree of oxidation is critical to the membrane-forming capacity of oxidized cholesterol solutions. Excessively oxidized and insufficiently oxidized cholesterol solutions failed to produce stable membranes. Pure cholesterol with its 3-hydroxyl group is an amphipathic compound and in an alkane solution should be able to form a black lipid membrane, but ordinarily does not [11].

Although cholesterol alone will not produce a stable bilayer membrane, a mixture of cholesterol plus a small amount of surfactant such as hexadecyltrimethylammonium bromide (HDTAB) will. An explanation for this is that HDTAB contributes enough extra polar groups so that the interfacial tension between the lipid membrane and its adjacent aqueous phases is reduced to a stable level [13]. The reduced interfacial tension promotes membrane stability by decreasing the thermodynamic free energy within the membrane, and this permits a more orderly and stable close packed arrangement of the cholesterol and HDTAB molecules [13]. If the membrane has too much surfactant, it is also unstable. Excess surfactant solubilizes cholesterol and disrupts the close orderly packing of the membane's molecules, because it reduces the interfacial tension to an unstable level [13]. There appear to be similarities between the concentration of surfactant and the degree of cholesterol oxidation in promoting membrane stability.

Oxidation converts cholesterol into a variety of compounds. The two most prevalent ones are 7-ketocholesterol and 7-hydroxycholesterol. These provide an additional polar group which might fulfill the role of a surfactant and reduce the interfacial tension to a stable level. The relationship between the specific capacitance and degree of oxidation seems to support this. Eqn. 12 indicates that membrane capacity depends on the dielectric constant which ordinarily would increase with an introduction of polarizable groups into the membrane phase. As the degree of oxidation time increases the specific capacity remains essentially constant (Tables I and II). This suggests that the polar groups produced by oxidation are at the interface of membrane and solution since specific capacity was not affected by an increase in cholesterol oxidation. These polar groups, if located at the membrane surface, could provide for a reduction in interfacial tension. With aging (Table III), specific membrane capacitance decreases and the least oxidized membranes have the highest specific capacitances. Eqn. 12 suggests therefore that either the dielectric constant decreases or that membrane thickening occurs with membrane solution age. Bilayer membranes made from phospholipids alone have a higher capacitance (approx. 2X) than those made which include decane and tetradecane [14]. This suggests that decane and tetradecane in bilayers contribute to bilayer thickening or a reduction in dielectric constant. Evidence supporting membrane thickening comes from studies showing that decane and tetradecane constitute a large portion of the thinned region of bilayer membranes [15].

The solubility of oxidized cholesterol in alkanes also contributes to its ability to form stable membranes. At room temperature, cholesterol is not very soluble in either *n*-octane or decane and tetradecane, but oxidized cholesterol is. Many of the lightly oxidized unstable membranes had white crystals in their Plateau-Gibbs borders. These crystals were probably of unoxidized cholesterol and occurred because of cholesterol's low solubility in the alkane solvent. Their presence seemed to contribute to the membrane's instability.

The role of the alkane solvent in bilayer lipid membrane stability appears important in the preparation of the solutions, because its boiling point determines the oxidizing temperature. The solutions prepared in *n*-octane refluxed at 126°C while the solutions prepared in decane and tetradecane refluxed at 198°C. The solutions prepared in *n*-octane were still lightly oxidized after 7 h of refluxing and required a month more of aging before they would form good membranes. The solutions prepared in decane/tetradecane only needed to be refluxed for 1.5 h, and they produced good membranes within 24 h after preparation. This indicates that higher refluxing temperatures appear to speed the formation of the necessary oxidation products.

The results in Tables I and II indicate that the membrane's total capacitance and resistance initially change with time during an experiment (approx. 20 min). However, the specific capacitance and resistance generally remain constant. This resulted from the thinned region of the membrane typically expanding during the experiment and indicates that continuous membrane diameter measurement is necessary in any critical bilayer lipid membrane study. White [6], who also worked with oxidized cholesterol, found that there was a 15% increase in the specific capacitance over the course of his experiments. He concluded that it was due to continued thinning of the membrane. Our results did not demonstrate this increase. The discrepancy between our data and his is not understood.

Acknowledgement

This research was supported in part by a grant from the National Institute of General Medical Sciences, National Institutes of Health.

References

1 Tien, H.T. (1974) Bilayer Lipid Membranes, p. 7, Marcel Dekker, Inc., New York
2 Mueller, P., Rudin, D.O., Tien, H.T. and Westcott, W.C. (1962) Circulation 26, 1167—1171
3 Tien, H.T. and Diana, A.L. (1968) Chem. Phys. Lipids 2, 55—101
4 Bergström, S. and Wintersteiner, O.J. (1942) J. Biol. Chem. 143, 503—507
5 Mueller, P., Rudin, D.O., Tien, H.T. and Westcott, W.C. (1964) in Recent Progress in Surface Science (Danielli, J.F., Parkhurst, K.G.A. and Riddiford, H.C., eds.), pp. 349—393, Academic Press, New York
6 White, S.H. (1970) Biophys. J. 10, 1127—1148
7 Jain, M.K., White, F.P., Strickholm, A., Williams, E. and Cordes, E.H. (1972) J. Membrane Biol. 8, 363—388
8 Tien, H.T. (1974) Bilayer Lipid Membranes, pp. 118—124 and 146—148, Marcel Dekker, Inc., New York
9 Babakov, A.V., Ermishkin, L.N. and Liberman, E.A. (1966) Nature 210, 953—955
10 Laüger, P., Lesslauer, W., Marti, E. and Richter, J. (1967) Biochim. Biophys. Acta 135, 20—32
11 Tien, H.T., Carbone, S. and Dawdowicz, E.A. (1966) Nature 212, 718—719
12 Bergström, S. (1943) Arkiv Kemi, Mineral. Geol. 16, 1—72
13 Tien, H.T. (1967) J. Phys. Chem. 71, 3395—3401
14 Benz, R., Fröhlich, O., Laüger, P. and Montal, M. (1975) Biochim. Biophys. Acta 394, 323—334
15 Pagano, R.E., Ruysschaert, J.M. and Miller, I.R. (1972) J. Membrane Biol. 10, 11—30