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Voltage-Induced Reflectivity Relaxation of Bilayer Lipid Membranes: On Changes of Bilayer Thickness

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Summary. Temporal and voltage-induced changes of reflectivity (R), the optical phase difference in transmitted polarized light, of tension and total capacity of bilayer lipid membrane (BLM) were studied. The membranes were mainly formed from total brain phospholipids (TP) in *n*-alkanes.

1) Reflectivity of "black" regions of films made of TP in decane and hexadecane decreases by several per cent with a time constant (τ_R) of about 30 min, whereas that of membranes with hexane and heptane does not depend on time (with an accuracy up to 1–2%). The BLM tension decreases appreciably in the course of time and reaches its steady-state value in tens of minutes after complete blackening of the membrane.

2) Under prolonged (up to tens of minutes) action of voltage (V) no R changes of BLMs with hexane, heptane, and hexadecane were revealed at a noise level of 0.2%. BLMs with decane usually respond to voltage application, first by a rapid (jump-like) and then by a slow decrease of R with a value spread from 0.2% to 3%.

3) With higher amplitude and temporal resolutions of the signal (signal averaging method) it can be seen that after voltage jump R decreases down to a new steady-state value: at $V=100$ mV, $\Delta R/R=-(2\text{–}4)\cdot 10^{-4}$ and $\tau_R\sim 0.1$ msec for BLMs from TP in heptane, and $\Delta R/R=-(3\text{–}6)\cdot 10^{-2}$ and $\tau_R\sim 2$ msec for BLMs from oxidized cholesterol in decane. It is shown in the latter case that the great value of $\Delta R/R$ is due to the contribution of invisible microlenses. In all the cases $\Delta R\sim V^2$.

4) It is concluded that at voltage jump a bilayer first becomes thinner due to volumic compression of its hydrocarbon core; then it spreads with a time constant of the order of 0.1 msec, getting thinner until a new equilibrium state is reached. Complete change of bilayer thickness is $\Delta h/h\sim -10^{-4}$ at 100 mV.

How significant are structural changes, particularly the change of thickness induced by electrical field in cellular and artificial membranes? This question arose primarily with the hypothesis that these changes possibly participate in the control of ion permeability of membranes. Investigations carried out on cellular and artificial membranes mainly with the use of optical methods showed (*see* reviews [10, 14]) that some

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potential-dependent structural changes really took place, but their values are insignificant. Thus, in the membrane of a squid axon during excitation the thickness and birefringence values change, according to our calculations [9, 10], by no more than 0.1 and 1.5 %, respectively. The change of these parameters in BLM appeared to be [9] smaller than those mentioned even at voltages of 0.2–0.3 V. It should be noted that even in the first work [3] dealing with the action of electrical field on BLMs, it was reported that their thickness changes by no more than 1 %. Independently, it was also shown [27] that changes of membrane capacity synchronized with alternating voltage, which occur due to electrostriction (at frequencies $\geqq 10$ Hz), are substantially less than 0.1 %.

At the same time some authors [2, 5, 6, 24–26], who determined specific capacity by measuring the total capacity and area of black membrane, reported that specific capacity increases by the order of percent and even of tens of percent [6] under the action of voltage of about 0.1–0.2 V. Does this mean that bilayer membrane becomes thinner by an appropriate value? A number of authors [20, 25–27] question this. Some mistakes could be expected in determination of the bilayer area due to the inaccuracy of estimating the real BLM contour [25, 27] as well as to the lack of information on BLM area occupied by invisible microlenses of lipid solution [20, 26, 27]. On the other hand, substantial difference between the optical and capacity measurements could be due to the fact that the former (and the capacity data of [27]) reflect short-duration (<0.1 sec) changes whereas the latter reflect long-duration (>10 sec) changes occurring in the membrane. Therefore, the difference in the results obtained by these two methods is possibly due to the fact that, under long action of voltage, more essential changes in BLM thickness occur which are connected with the excess of solvent from bilayer into lenses and annulus, as supposed by Requena *et al.* [20]. But if this is an excess of the solvent, it is logical to suppose that its value depends on the "age" of BLM. With "age", BLM parameters change, and this is justified by the following: increase of specific capacity [5, 6, 16, 24], change of membrane content [12], and weakening of electro-optical effects [9]. It is natural that the action of voltage on the membrane when it is in stable (equilibrium) state is of greatest interest.

The present paper is devoted to the investigation of short- and long-duration (tens of minutes) effect of voltage on BLMs of different content. The dependence of rate with which black membrane reaches its equilibrium state on the solvent type is also presented. The equilibrium state was judged according to stabilization of values of BLM thickness and tension on a constant level.

Materials and Methods

Optical Technique

The change of BLM thickness in the course of time was judged according to the change of intensity of the light reflected by the membrane. The light is polarized perpendicular ($\perp$) to the plane of incidence. Corresponding equations describing the quantitative relation of membrane reflectivity with its thickness and the reflective indexes, as well, with their small alterations have been obtained [11]. BLM is considered as a homogeneous anisotropic film with an optical axis perpendicular to its surface and with some effective values of thickness h and the main reflective indexes n_0 and n_e. The membrane divides two isotropic mediums with the refractive index n_I. Taking into account the real parameters of both BLM and water medium: $h < 10$ nm, $n_o \approx n_e < 1.5$, $n_I \geqq 1.333$, for the angles of light incidence, $\theta_1 < 50°$, the following simple expression for the reflectivity R of the membrane is truthful with an accuracy of 2%:

$$R = r^2 \cdot \beta^2, \tag{1}$$

here $\beta = 4\pi h \lambda^{-1}(n_2^2 - n_1^2 \sin\theta_1)^{1/2}$, r is the Frenel's reflection coefficient, and n_2 is the refractive index of the membrane. The values r and n_2 depend on the plane of light wave polarization. Figure 1 demonstrates the values of R, r^2 and β and their dependence on the angle of incidence and light polarization for lecithin membrane in water solution with $n_1 = 1.334$.

In the most interesting case, when there is light wave with the electrical vector perpendicular to the plane of incidence, the following expression for the relative change of membrane reflectivity has been obtained [11]:

$$\frac{1}{2}\frac{\Delta R_\perp}{R_\perp} = \frac{\Delta h}{h} + \frac{2}{1 - n_1^2/n_0^2} \cdot \frac{\Delta n_0}{n_0}. \tag{2}$$

The coefficient before the second summand is more than 10. Thus, for lecithin BLM ($n_0 = 1.46$) in the salt solution with $n_1 = 1.334$, this coefficient is 11.8. From Eq. (2) it is seen that to estimate two unknown variables $\Delta h/h$ and $\Delta n_0/n_0$ it is necessary to measure $\Delta R_\perp/R_\perp$ at two values of n_1, i.e., in the mediums of different content. However, a rough estimation of maximal value of $\Delta h/h$ can be made by the only measurement of $\Delta R_\perp/R_\perp$. This estimation is based on a reasonable assumption that in a common case neither summand in the right part of Eq. (2) can compensate the other with a high degree of accuracy. It means that $|\Delta h/h|$ is of the same order as $|\Delta R_\perp/R_\perp|$ or less.

To measure intensity of the light reflected from BLM, the optical scheme used did not principally differ from that described previously [9]. The light from a source having passed through the polarizer was focused by a condenser in the membrane plane, forming the light spot, with the diameter several times less than that of BLM. The beam of light reflected by BLM was directed by the objective through an analyzer to the photomultiplier. In the plane, where the objective gave the image of the membrane, an additional field diaphragm was mounted which let only the light from the central illuminated part of BLM pass through. Due to this, the light from the lipid annulus of the membrane and the artefact optical signals connected with its change were eliminated.

To reveal a weak signal from the noises, a signal average designed on the base of Nokia multichannel analyzer LP-4050 was used in the experiments on the action of short electrical impulses on BLM. As a source of light, the helium-neon laser with $\lambda = 633$ nm was used. The response of the membrane to short voltage impulses was also studied in transmitted polarized light, using the technique described earlier [9]. The change of

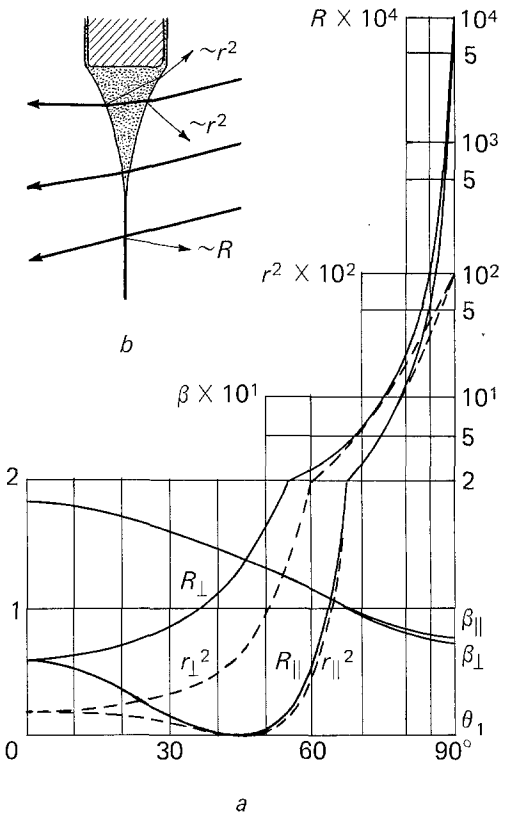

Fig. 1. (*a*): Theoretical dependence of reflectivity (R), reflection coefficient (r), and phase difference (β) of lecithin BLM in water solution with $n_1 = 1.334$ on the angle of incidence θ_1 and on the character of light wave polarization: parallel (||) and transversely ($\perp$) to the plain of incidence. BLM parameters according to [13], $h = 6.2$ nm, $n_0 = 1.46$, $n_e = 1.48$. Wave length is 633 nm. Along the ordinate from 0 to 2, the scale is linear, then it is logarithmic. (*b*): The scheme of light reflection and refraction by planar bilayer and annulus

phase difference γ, arising in the membrane due to birefringence and to the contribution of the rays reflected for the second time [7], was registered. The angle of incidence was $\theta_1 = 45°$. The change of light transmission through the membrane was registered without any analyzer.

While studying the slow processes (longer than 1 sec), the registration of photomultiplier current was carried out directly by a high sensitive recorder. In these experiments the prolonged stability of the light flux was important; that is why the source of the light was a 90-W halogen-tungsten lamp with a green glass filter ($\lambda_{max} = 540$ nm; half-width of transmission band = 90 nm). The lamp was supplied by stabilized source of constant current. Relative threshold of the set-up sensitivity to the change of light flux reflected by BLM was not greater than 0.1 %.

In optical experiments two types of chambers for manipulation with BLM were used. The first chamber had a partition made of black glass with the aperture for the

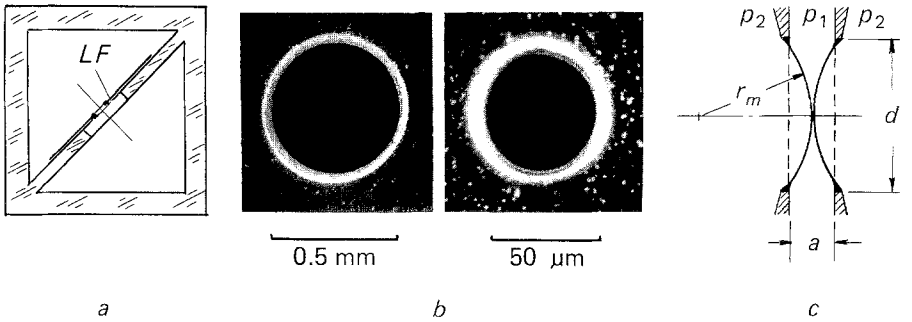

Fig. 2. (*a*): A glass chamber for optical experiments with BLM (top view, in section). The inner size of the chamber is $20 \times 20\,mm^2$, the diameter of the hole in a glass partition is 6 mm. (*b*): View of holes in Lavsan film (LF) 10 μm in thickness made by a hot needle (Dark-field illumination). LF film with a hole for a membrane is glued to a partition of the chamber (*a*) by vacuum grease. (*c*): Scheme of measurement of membrane tension (σ) by pressure difference $\Delta p = p_2 - p_1$, which is necessary for contact between membranes

membrane and its two walls constructed as optically regular semispheres. The diameter of the aperture was 2.5 mm, the wall thickness near that – 0.2 mm. This chamber allowed us to carry out measurements on the membrane in a wide range of angles of light incidence. A rectangular glass chamber (Fig. 2*a*) with a glass partition was used to measure the changes of BLM reflectivity at $\theta_1 = 45°$ [then $R_{\parallel} \ll R_{\perp}$ (Fig. 1)]. The membrane was secured on a 0.5 to 0.9-mm diameter lavsan film (polyethyleneterephthalate, 10 μm thick) aperture, glued by vacuum grease to a glass partition. The aperture in lavsan film was made by a heated needle, so that it was a regular round shape with smooth round edges (Fig. 2*B*). (This is an ideal aperture for securing a bilayer from two monolayers [5]). The apertures for the membranes in both the chambers were lubricated by lipid solution before adding the aqueous solution.

Measurement of BLM Capacitance

Measurement of the total membrane capacitance (C) and its slow changes were carried out by a bridge method at the frequency of 1 kHz and efficient voltage on the membrane of about 15 mV. To carry out continuous recording of the capacitance change $\Delta C(t)$, the bridge was used in the regime of disbalance. In this case the change of output signal of the bridge reflects the value of ΔC and its sign. In this scheme an independent supplement of a constant and low frequency voltage (up to 90 Hz) on the membrane was carried out. A relative threshold of the scheme sensitivity to the capacitance change was not greater than 0.2 %. Voltage was supplied to the membrane with the help of Ag – AgCl electrodes or, in the case of asymmetrical solutions, liquid (agar) bridges.

Measurement of Membrane Tension

The method used to measure membrane tension (σ) is based on the dependence of the pressure difference Δp sagging the membrane, on the values σ and r_m: $\Delta p = 2\sigma / r_m$ where r_m = radius of the membrane curvature. In the scheme used for measurement of σ,

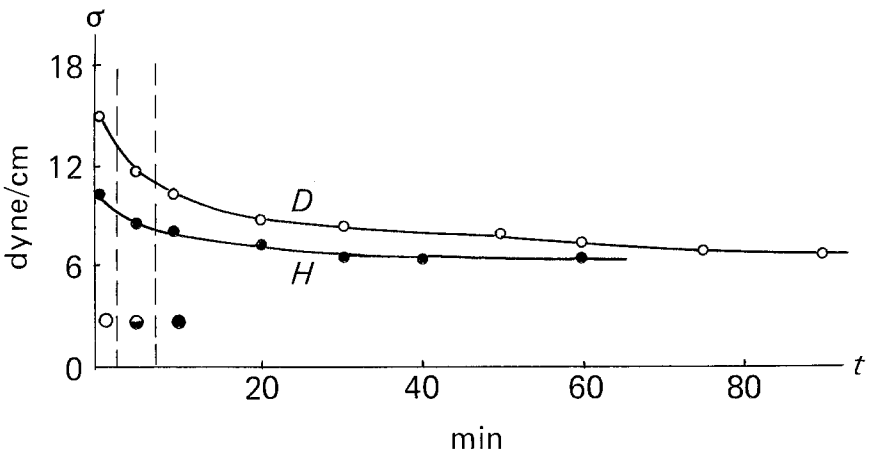

Fig. 3. Time dependence of tension of membranes from total brain phospholipids in *n*-decane (*D*) and *n*-heptane (*H*). The period of blackening is marked by broken lines (10 mM NaCl, pH 5.7)

the value r_m is a constant one. r_m is determined, as it is seen from Fig. 3, by diameters of the apertures and the distance between partitions ($a = 0.3$–0.5 mm, $d = 1.0$–1.5 mm). The pressure difference Δp was obtained by microsupplement of an additional volume of the solution to the external compartment of the chamber [15]. The procedure of measurement was the following: The membranes initially plane and parallel to each other were put in contact on a small area (in the point). The established contact was observed in reflected white light with the help of a microscope with a low magnification. The additional volume of the solution was measured, and the membranes were again separated to prevent fusion during a long contact (the fusion in the solutions of monomonovalent salts occurs at concentration more than 10 mM [8, 15]). By establishing periodical contacts between the membranes, the dependence $\sigma(t)$ was obtained.

Materials

Total bovine brain phospholipids were extracted according to the well-known Folch's method; egg lecithin from Merck was used. Oxidized cholesterol was obtained by blowing oxygen through a boiling solution of recrystallized cholesterol in purified *n*-octane for 8 hr. The normal alkanes (analytical grade) were used without additional purification. Membranes were usually formed from 20 mg/ml of lipids in *n*-alkanes.

Results

The Change of BLM Thickness and Tension in the Course of Time

The behavior of BLM is judged according to bilayer reflectivity. For the experiments a chamber with lavsan film was used (Fig. 2*a*). The

diameter of a light spot was no more than 0.2 of that of the aperture. The measurement began from the moment the area of the spot was completely occupied by a black membrane (that was earlier than when the membrane became black totally); blackening usually proceeded for 10–15 min. The experiments were carried out on the membranes from the total phospholipids in different *n*-alkanes in 0.1 M NaCl solution. The specific capacity of black membranes was 0.37–0.38 $\mu F/cm^2$ in the case with heptane and 0.56–0.58 $\mu F/cm^2$ with hexadecane. The $R_\perp$ value of hexane and heptane BLMs is constant (with accuracy of 1–2%) from the moment of their formation. The reflectivity of decane, hexa-, and heptadecane membranes decreases in the course of time approximately by 8% and 4%, respectively, with the time constants in the range of 20–30 min (working temperature for decane BLM 20 °C, for hexa- and heptadecane BLM, 35 °C). Decrease of $R_\perp$ can be connected with that of both the thickness h and refractive index n_0 of the membrane (Eq. (2)). But n_o may decrease only when the part of the solvent in the bilayer increases, since the refractive index of the solvent is less than that of lipids. However, the greater mobility and compressibility (*see below*) of the membrane in the early period justify greater content of the solvent at that time. Thus, due to decrease of the solvent part, the membrane must become thinner and the refractive index greater. Correspondingly, in Eq. (2) the summands are of different signs. Since $\Delta h/h$ has the same sign as $\Delta R_\perp/R_\perp$, then $|\Delta h/h| > |0.5 \cdot \Delta R_\perp/R_\perp|$. But it is hardly probable that this inequality is great because it is unlikely that the summands of Eq. (2) have close absolute values. From this it follows that black membranes become thinner only by some percent in the course of time.

Dependence of membrane tension on the course of time was measured for the membranes from common phospholipids of brain with *n*-heptane and *n*-decane. The measurement began when the membrane was colored. Characteristic dependences $\sigma(t)$ are given in Fig. 3. Initial values of σ and the relaxation time vary greatly from one membrane to another, but final values of σ (6 dyne/cm) repeat well. Usually tension for heptane membrane gets its steady state in 20–30 min, and for decane, in 30–60 min. Since BLM tension is determined by surface tension of two lipid monolayers on the annulus (on boundaries of oil/water), then the surface tension of monolayers decreases as well. The latter is connected with those facts, that density of the monolayer formed by absorption of lipid molecules from the solution increases with time, and tension, respectively, decreases. The process of absorption seems to occur as slow, as high as the viscosity of the solvent, and as low as the diffusion coefficient

of lipid molecules in the solution. That is why tension of decane membrane is set slower than that of heptane.

Usually when the membrane becomes completely black, the change of the annulus width goes on in the course of time. The case of complete disappearance of the annulus is of particular interest. Fettiplace *et al.* [16] observed that BLM from lecithin + *n*-hexadecane in the course of time reaches an area not only equal to that of the aperture, but much larger. In our experiments with the aperture in lavsan film, the BLM from total phospholipids + *n*-hexadecane had not gone over the edges of the aperture, but the annulus disappeared completely in half an hour. In this case BLM was in the mid-plane of the hole and its capacitance C was not changing any longer. Probably, existence of a thickened smooth edge of the aperture provides the stable position of BLM. Heptane and decane BLM usually have an annulus 50–80 μm wide in such an aperture.

BLM Reaction to Voltage. Slow Processes

In optical experiments on observation of BLM reaction to voltage impulses, it has been revealed that a long-duration effect of a constant voltage on the membrane leads to its structural asymmetry: it becomes sensitive to the sign of voltage impulse. Therefore, to observe the effects of long duration of voltage an alternating voltage was supplied to BLM at a frequency of 90 Hz. In a number of experiments a constant voltage equal to the effective value of the alternating one ($V_\sim$) was supplied for comparison. There was no difference (according to the change of total capacitance) in BLM reaction. The frequency of alternating voltage (90 Hz) was chosen for two reasons. First, at this frequency annulus as well as lenses stop "to breathe" [22, 27], and, second, this voltage has no effect yet on the result of membrane capacitance measurement made at the frequency of 1000 Hz. The experiments were carried out on membranes from total phospholipids with different solvents. The changes of BLM reflectivity and total capacitance were registered.

Our system for optical measurement allowed us to register slow changes of reflectivity $\geqq 0.2\,\%$. However, even at such a high sensitivity one failed to discover the change of $R_\perp$ for hexane and heptane membranes at $V_\sim = 0.2$–0.25 V; and for those of hexadecane (within 1 hr after blackening) at 0.1 V. An example of $R_\perp(t)$ record is given in Fig. 4*c*. From the results obtained, it follows that in the case of hexane, heptane,

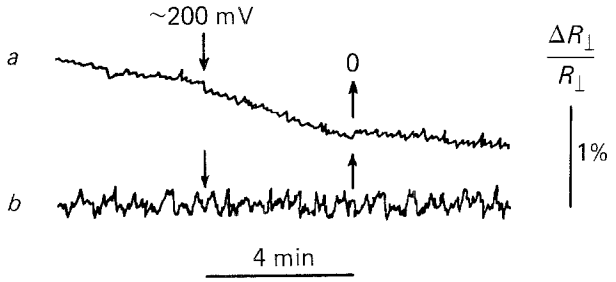

Fig. 4. Effect of long-duration action of alternating voltage (200 mV, 90 Hz) on reflectivity of BLM from total brain phospholipids in *n*-decane (*a*) and *n*-heptane (*b*). Voltage was applied within 20 min after complete blackening of the membranes (0.1 M NaCl, 20 °C)

and hexadecane the BLM thickness changes, if really it is so, under the action of 0.1–0.2 V by no more than some tens of percent.

But decane BLM behavior is different in character. In response to a switch of $V_\sim$, at first $R_\perp$ usually decreases quickly (by jump), and then it decreases slowly (Fig. 4*a*). When $V_\sim$ is switched off, on the contrary, $R_\perp$ increases by jump by the same value, and incline of the curve $R_\perp(t)$ changes into positive direction. The values of jump-shaped and slow changes of $R_\perp$ vary greatly from one membrane to another, and even in the same membrane, in the course of time. Jump-shaped changes of $R_\perp$ (at $V_\sim = 0.2$ V) were about 0.1 to 1.5%; slow ones (during 5–10 min) were either absent at the level of 0.2–0.3% or reached 2–3%. Greater changes usually are connected with the presence of more lenses in BLM and even with the appearance of new ones under the action of electrical field, as it was also stated by Requena *et al.* [20].

Measurement of total capacitance C was carried out simultaneously with optical ones on the same membranes. A typical example of C change in due course is given in Fig. 5. From the curves it is seen that there are processes, at least, with three characteristic times: <1 sec (jump), ~ 0.5 min, and ~ 5 min. Usually the value of ΔC at $V_\sim$ switched on is higher than at switched off. But when $V_\sim$ is switched on for many times this difference decreases. The value of C changes is significantly dependent on the type of lipid solvent. Thus, at $V_\sim = 0.2$ V a quick jump of C of heptane BLM does not exceed 1% and of decane BLM reaches 1.5–10% (the width of annulus in both cases is 10–15% of the aperture diameter). Slow changes of C (during 5 min) have the values 1–1.5% and 5–10%, respectively. Capacitance of hexadecane BLM without annulus on the response of $V_\sim = 0.1$ V increases only by 0.2% with the time

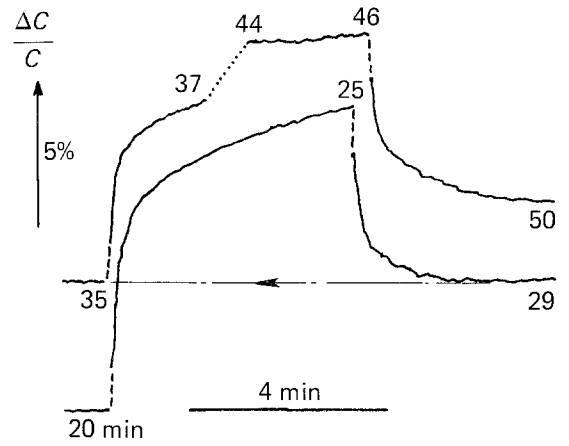

Fig. 5. Relative change of a total capacitance (C) of the membrane from total brain phospholipids in n-decane under the action of alternating voltage (200 mV, 90 Hz). The voltage was supplied from 20 until 25 min and from 35 until 46 min after complete blackening of the membrane. Capacitance jumps, shorter than 1 sec, are marked by a broken line (0.1 M NaCl, 20 °C)

constant ~1 min. The character and value of the change of C are greatly affected by size and geometry of the aperture and other reasons. Thus, the value ΔC of membranes placed on the aperture in glass 2.5 mm in diameter (*see Methods*) was essentially higher, and the changes of C lasted longer. Different values were obtained by other authors [5, 6, 21]. Increase of C under the action of potential difference occurs due to spreading of a bilayer, since the thickness of the latter, as it is shown above, hardly changes.

Decane membrane, as it follows from our and other data [4, 6, 12, 24, 26], possesses an extremely variable and labile structure. If we use as a solvent n-alkans with essentially a smaller or, on the contrary, greater number of hydrogen atoms than that of decane, then BLM obtained are more free from heterogeneity (lenses and microlenses) and respond more weakly to the electric field.

BLM Reaction to Voltage. Quick Processes

The change of BLM reflectivity was studied when rectangular and saw-toothed voltage impulses were supplied. The latter were used to obtain the dependences of $\Delta R/R$ on the voltage V. $\Delta R/R$ was measured at two different light polarizations with the aim of revealing the contri-

Table 1. Change of reflectivity (ΔR) of BLM of different content under the action of a short (<0.1 sec) voltage impulse −100 mV [a]

Content of lipid solution	Aqueous medium	$\Delta R/R$	τ_R (msec)
Total phospholipids + *n*-heptane	0.1 M NaCl	$-(2-3.5)10^{-4}$	0.15–0.2
	4 M NaCl,	$-(1.8-2.5)10^{-4}$	0.2
	$n_1=1.368$	$-(8.1-8.3)10^{-4}$	0.2[b]
Total phospholipids + oxidized	0.1 M NaCl	$-(2-3)10^{-4}$	0.1
cholesterol (1:1) + *n*-heptane	0.1 M NaCl + glycerine $n_1=1.400$	$-(2.5-4)10^{-4}$	0.5[c]
Egg lecithin + *n*-heptane	0.1 M NaCl	$-(2.5-3.5)10^{-4}$	0.1–0.15
Egg lecithin + *n*-decane	3 mM KCl	$-(2-4)10^{-3}$	2–5[d]
		$-(2-5)10^{-4}$	1–2
Oxidized cholesterol + *n*-decane	0.1 M NaCl	$-(3-6)10^{-2}$	2

[a] $\Delta R/R$ = value of relative reflectivity change, τ_R = time of relaxation, n_1 = refractive index of aqueous solution, pH ≃ 7, 19–20 °C.
[b] At 200 mV.
[c] The process of relaxation is of complicated character (Fig. 6*d*).
[d] During first 10–20 min after a complete blackening of the membrane.

bution of optical anizotropy of the membranes [11]. However, due to the dispersion of the results, we failed to establish a difference between $\Delta R_{\parallel}/R_{\parallel}$ and $\Delta R_{\perp}/R_{\perp}$. That is why, in a corresponding column of Table 1, the averaged values of $\Delta R/R$ are given. Examples of optical signals obtained from different BLMs in various mediums are given in Fig. 6. In response to rectangular impulse of voltage, the reflectivity of BLM in some time (relaxation period) gets its steady state. This change of reflectivity ($\Delta R/R$) is shown in Table 1.

Reflectivity of all the membranes, as seen from Table 1, decreases under the action of electrical field. However, this decrease (except for a peculiar case of BLM from oxidized cholesterol with *n*-decane) (*see* Tables 1 and 2 and Fig. 6*b–c*), is insignificant: $\Delta R/R=-(2-5)\cdot 10^{-4}$. Thus, the change of BLM thickness is also insignificant. The time of relaxation, determined by the beginning of the optical signal impulse when decane is used, is higher by an order of magnitude or more than in the case of heptane (1–3 msec and 0.1–0.2 msec, respectively). Also, it is found that, during the first 20–30 min after complete blackening and by the time of relaxation, the optical signals from decane membranes are higher in value. The dependency $\Delta R(V)$ is of parabolic character for all the membranes studied (Fig. 4*a–b*), i.e., $\Delta R \sim V^2$.

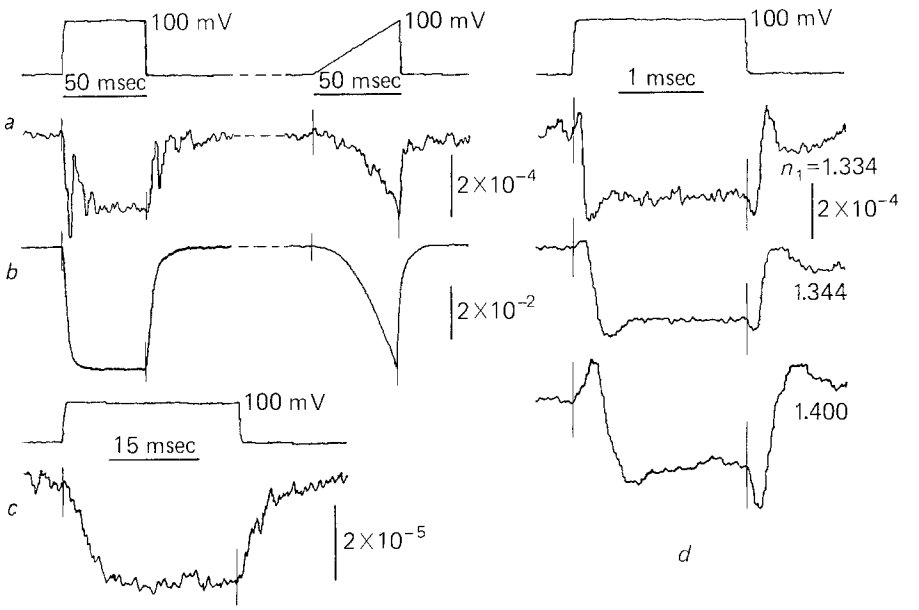

Fig. 6. Examples of reflectivity (*a*, *b*, *d*) and light transmission changes (*c*) of different BLMs under the action of voltage impulses with amplitude of 100 mV. Voltage impulses are seen above the corresponding curves of optical signals. Membranes from total brain phospholipids in *n*-heptane (*a*), from oxidized cholesterol in *n*-decane (*b*, *c*) and from the mixture of both lipids (1:1; w/w) in *n*-heptane (*d*). Water solution: 0.1 M NaCl, pH 7. To increase the refractive index of the medium (*d*, two bottom curves), glycerine was added to the solution

Table 2. Change of optical phase difference ($\Delta\gamma$) and BLM light transmission under the action of rectangular voltage impulse (V)[a]

Content of lipid solution	Aqueous medium	V (mV)	$\Delta\gamma$ (seconds of arc)	τ_γ (msec)	Change of light transmission
Total phospholipids + *n*-heptane	0.1 M NaCl	200	$<0, > -0.1$	0.2	is absent at the level 10^{-7}
Oxidized cholesterol + *n*-heptane	0.2 M NaCl	100	is absent at the level 0.1		is absent at the level 10^{-6}
Egg lecithin + *n*-decane	2 M KCl	100	0.03 – 0.08	2–3	$-(1-2)10^{-6}$
Oxidized cholesterol + *n*-decane	0.2 M NaCl	80	0.9 – 1.6	2	$-(1-3)10^{-5}$

[a] τ_γ = time of relaxation of phase difference. Angle of light incidence to the membrane $\theta_1 = 45°$. pH ≃ 7; 19–20 °C.

With the aim of finding out from Eq. (2) the values $\Delta h/h$ and $\Delta n_o/n_o$, the measurement of $\Delta R_\perp/R_\perp$ was carried out in the solutions with different reflective indexes n_1. In order to increase n_1, glycerine was usually added to the solution. A characteristic set of curves $\Delta R_\perp(t)$ for heptane BLMs is given in Fig. 6*d*. Transitional processes at switch on and off of the voltage are of complicated multicomponent character. The curve of transitional process when V is switched off is a mirror image of that at switched on. The shape of the curve indicates that there are two processes: a quick and a slow one in BLM which cause ΔR of opposite signs. The slowing down of the second process with the increase of n_1 is connected, probably, with the increase of viscosity of the glycerine solution. As for true kinetics of a quick process which follows the voltage jumps, it is not revealed here due to scheme limits; the time constant of capacitance overcharge was $30-50$ μsec. Steady-state value of $\Delta R_\perp/R_\perp$ in the range of experimental error is independent of n_1. It means, in correspondence with Eq. (2), that after a transitional period the refractive index of the membrane (n_o) returns to its initial value. An attempt to use sucrose instead of glycerine was unsuccessful: BLMs formed in concentrated sucrose solutions ($n_1 \leqq 1.415$) are covered by pale spots. That is why, probably, the fronts of optical signals were delayed up to tens of milliseconds, amplitude of $\Delta R_\perp/R_\perp$ increased by 1–3%, and optical responses on voltage impulses of different signs appeared to be asymmetrical.

Even earlier [9], the effect of lenses (bright points) on the optical signal (in transmitted light) from BLM under the action of electrical impulse was especially studied. It was found that when there were ten or more lenses in lightened BLM area it led to a significant increase in amplitude of the optical signal, and the relaxation time τ_γ increased by some milliseconds and more. The larger the lenses, the greater the τ_γ. That is why in order to obtain the optical signal from a real bilayer, we carried out the experiments only on BLM without visible lenses.

In heptane BLMs, mechanical damped oscillations excited by electrical impulses appear which cause corresponding oscillations in the intensity of both BLM light transmission [9] and reflectivity (Figs. 6*a* and 7*a*). In the case of reflectivity, they are expressed more clearly. The oscillations begin with some delay after the voltage jump (Fig. 7*a*). The time of delay of oscillations begins to decrease and the amplitude increases when light spot is shifted from the center of the membrane to its periphery. This points to the fact that an elastic wave caused by a sharp shift of the annulus edge due to the tension change of bilayer propagates

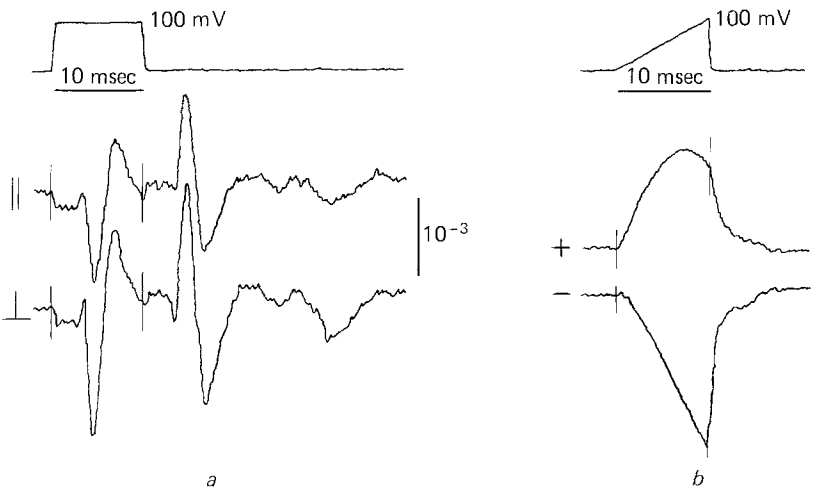

Fig. 7. Reflectivity change of BLMs from total phospholipids in *n*-heptane (*a*) and from oxidized cholesterol in *n*-decane (*b*). (*a*): Example of mechanical oscillations in BLM. The curves are obtained at two light polarizations. (*b*): Example of optical signals from asymmetric membrane; asymmetry is obtained by pH difference on both sides of the membrane (pH 3 and 9, 2 mM NaCl). Optical signals for voltage impulses are of different signs

along BLM from the edge to the center [17, 19, 22]. In order to clarify whether this wave is longitudinal or transversal, the measurements were carried out at two different light polarizations. In the case of transversal wave, the definite membrane sites would change their position from an averaged one leading to a corresponding change of light incidence angle (θ_1) (on this site). Then, at $\theta_1 = 30°$, as it is shown in Fig. 1, the change of θ_1 would cause the increments of $R_\perp$ and $R_\parallel$ of different signs. However, in the experiments (Fig. 7), oscillations of $R_\perp$ and $R_\parallel$ in phase were observed. From this it is inferred that the main contribution is made by the change of h and n_2 but not by θ_1, as we have supposed earlier [9]. This feature is characteristic of a longitudinal wave. When the membrane is not a plane, a transversed wave arises, as well.

As mentioned above, the value of ΔR is proportional to that of V^2 and, therefore, does not depend on a voltage sign. But this fact concerns only structurally symmetrical BLMs. Asymmetrical membranes are obtained in some cases and the magnitude of their optical signal depends on a sign of the voltage impulse supplied. In particular, appreciable structural asymmetry in BLM is caused by a long-duration action of a constant voltage and pH difference on each side of the membrane. Thus,

small constant voltage, if it is switched steadily, but not only for the time of a test impulse, leads to more appreciable asymmetry of optical signals. At different pH on each side of BLM the optical signals on voltage impulses of different polarity change as if some constant voltage is supplied to BLM. A characteristic example is given in Fig. 7*b*. It is seen that the top of the parabola (middle curve) is shifted by ~ 70 mV. Structural asymmetry of BLMs in both cases must be somehow connected with the density difference of surface charges of both monolayers. The tension of each monolayer [8] and, probably, lipid composition depend on the density of these charges.

Discussion

Let us consider the successive events developing in BLM caused by a voltage jump. Simultaneously with a voltage jump, compressive force increases ($\sim V^2$) and tension of a bilayer decreases ($\sigma_o - \sigma_v \sim V^2$) [17, 19, 22, 27]. Compressive force acts only transversely to the bilayer and does not prevent its elastic spreading. As a result, the bilayer must become not only thinner, but spread as well. Since bilayer spreading is connected with its sliding in viscous liquid, this process proceeds slower than thinning. Thus it is logical to conclude that at first a bilayer must undergo a volumetric compression (due to Δh) which decreases during spreading.

Now, in order to see how these events are elucidated by the results of optical experiments, let us analyze the sets of curves (Fig. 6*d*) obtained on the bilayers practically without microlenses. Analyzing the signal $\Delta R_\perp / R_\perp$ according to Eq. (2), we use, as reported earlier [11], the relation of n_o to h. The change of refractive index (n_o) is composed by two constituents: One is connected with the change of density of membrane substance, and the other with the change of optical polarizability of the molecules. Between these two components there is a certain relation which follows from the equation of Lorentz-Lorenz: $(n^2-1)/(n^2+2) = 4\pi Na/3$, where N = a number of molecules in the volume unit and a = effective optical polarizability of the molecules. Under the action of external force (electrical field), an elementary volume of the membrane may change the thickness as well as the occupied area S, but NhS remains constant. Therefore, $(n^2-1)/(n^2+2) \sim a/hS$, from where we shall obtain for our case:

$$\frac{6n_o^2}{(n_o^2-1)(n_o^2+2)}\frac{\Delta n_o}{n_o}=\frac{\Delta a_o}{a_o}-\frac{\Delta h}{h}-\frac{\Delta S}{S}. \tag{3}$$

In particular, for BLM with $n_o=1.46$ the coefficient in the left part of Eq. (3) is equal to 2.7. Taking into account this coefficient for the mediums with $n_1=1.333\div1.4$, we obtain from Eqs. (2) and (3)

$$\frac{\Delta R_\perp}{R_\perp}=(8.2\div15)\cdot\left[\frac{\Delta a_o}{a_o}-\frac{\Delta S}{S}-(0.76\div0.87)\frac{\Delta h}{h}\right], \tag{4}$$

where the first figures in brackets correspond to $n_1=1.333$.

Change of a_o is connected with Kerr's effect. To estimate the value $\Delta n_o/n_o$ connected with Δa_o, let us assume that the electrical field is concentrated in the hydrocarbonic part of a bilayer (h_c in thickness) and, therefore, it has a small effect on the polar regions of a bilayer. Moreover, they make up a small part of the total thickness. That is why we use Kerr's constant value (B) for normal hydrocarbons [23]. For hydrocarbons $n\text{-}C_7H_{16}\div n\text{-}C_{18}H_{38}$ $B=(8.5-18)\times10^{-14}$, that is of the order -10^{-13} cm^{-1} (V/cm)$^{-2}$. From two equations for Kerr's effect [23] (which do not concern the effect of electrostriction) we find $\Delta n_o/n_o=(n_{ov}-n_o)/n_o=-\lambda BV^2/3n_o h_c^2$. For $V=0.1$ V, at $n_o=1.46$, $\lambda=550$ nm, $h_c=5$ nm, and $B=10^{-13}$ cm^{-1} (V/cm)$^{-2}$, we obtain that $\Delta n_o/n_o\sim5\times10^{-8}$. Such a change of n_o causes $\Delta R_\perp/R_\perp\sim-10^{-6}$; that is less, at least by an order, than the noise level in the recordings of Fig. 6*d*. Thus, $\Delta a_o/a_o$ does not make any appreciable contribution to the signal $\Delta R_\perp/R_\perp$.

Now let us consider the contribution of electrostriction. The value of the force (pressure) pressing the bilayer $\Delta p=C_m V^2/h_c$. For $V=0.1$ V and $C_m=0.35$ μF/cm^2 we obtain $\Delta p=0.07$ atm. Compressibility factor $\left(K_T=-\frac{1}{v}\frac{\partial v}{\partial p}\right)$ for hydrocarbons is $\sim10^{-4}$ atm^{-1}. From this we obtain relative change of bilayer volume $\Delta v/v=7\times10^{-6}$. At the first moment after the jump, as mentioned above, a bilayer becomes thinner; this means that $\Delta h/h=\Delta v/v$. In this case from Eq. (4) we find that $\Delta R_\perp/R_\perp$ depending on n_1 must have values approximately from 0.5×10^{-4} to 10^{-4}. From the curves (Fig. 6) it is seen that estimated values of $\Delta R_\perp/R_\perp$ are close in magnitude and sign with those measured. It should be noted that in a given case $R_\perp$ increases (as it follows from Eq. (3)) at the cost of $\Delta n_o>0$.

After initial increase, $R_\perp$ falls down to some steady-state value which does not depend on n_1 in the range of error of measurements. So on the

basis of Eq. (2) we have drawn the conclusion that the refractive index n_o of a bilayer increased by a jump in the beginning of a transitional period then returns back to its initial value (at $V=0$). Since $\Delta a_o/a_o$ is negligibly small it means that $\Delta v/v=\Delta h/h+\Delta S/S=0$. From Eq. (2) for a steady-state value $\Delta R_\perp/R_\perp=2\times10^{-4}$, taking into account that $\Delta n_o=0$ we find $\Delta h/h=-10^{-4}$ and, respectively, $\Delta S/S=10^{-4}$. Thus, while passing to steady state after the jump of V (=0.1 V), the bilayer becomes thinner by 0.01 % and spreads to the diameter by 0.005 %. As a result, specific capacity must increase by 0.02 %; that is in good agreement with the data of Wobschall [27] on the capacity changes at frequencies $\geqq$10 Hz. It should be noted that the process of bilayer spreading, as seen from the curves $R(t)$ (Fig. 6), are of quick-damping oscillating character (near the steady state).

Now we shall consider the behavior of lenses and annulus and their effect on the results of optical and capacitance measurements. Decrease of bilayer tension at voltage jump causes the change of a contact angle θ between bilayer and liquid phase – lenses and annulus – that in its turn leads to the change of the shape of these elements [17, 19, 22]. Some time after V jump, the lenses pass into a new equilibrium state becoming more convex but less in diameter [22]. The time of lens relaxation depends on its size. For small lenses (<10 μm in diameter) this time, (according to optical measurements) does not exceed 10 msec. But lenses with diameter about 100 μm do not follow the voltage even at a frequency of 10 Hz [22]. As for the time of relaxation – 1–2 msec (Tables 1 and 2) – seen in optical signals obtained from BLM without visible lenses, it may be due to invisible microlenses (<1 μm) revealed in BLMs by electron microscopy [1]. Existence of microlenses is justified by the following facts:

a) Decrease of BLM light transmission simultaneously with a fall of reflectivity (Table 2, Fig. 6*c*). Such inconsistence can be explained only by increase of light scattering of a bilayer and, therefore, by the existence of some microheterogeneity in BLMs or its appearance under the action of electrical field. Especially, there is a great number of microlenses in the membranes from oxidized cholesterol + *n*-decane. That is why they have a specific silver glitter in reflected light, and the change of their reflectivity is two orders of magnitude higher than that of heptane BLM (Table 1), since it is completely due to the change of light-scattering by microlenses.

b) In a region of contact, two decane BLM begin to fuse at a separate point at the distance between the bilayers of about 30–40 nm

[15]. The facts prove that at first two microlenses from opposite bilayers fuse [8]. It should be noted that the thickness of the microlense (1 μm in diameter, with a contact angle of 2°) is about 25 nm.

Unlike the lenses where relaxation processes are finished in 10 msec, in an annulus they occur for 10 min as shown by the dependence $C(t)_v$ (Fig. 5, [5, 21, 25, 27]). The reason for this may be explained by the large surface of the annulus, which is also on the surface of the partition outside of the aperture. A sharp decrease of bilayer tension under the action of voltage and increase of the contact angle lead to displacement of the liquid phase, not only in the visible annulus part in the aperture but in its "wings" on the partition as well. The process of annulus change as a whole can be divided into three stages. In the first stage, lasting for tens of milliseconds, the thinnest part of an annulus sharply decreases. This edge becomes thicker. Change of annulus geometry occurs more slowly. Since the contact angle has increased and the thickness of a partition edge near the aperture is constant, then the volume of an annulus in the aperture must decrease. The process of an output of solvent excess from the annulus itself into its "wings" leads to a gradual increase of BLM area. Increase of C with a characteristic time ($\sim$0.5 min) corresponds, probably, to this process (*see above* and [4, 6, 21]). At last, the increase of liquid phase volume in the "wings" leads to their spreading over the partition. That is why C does not return to its initial value, but remains more than that when the voltage is switched off (Fig. 5 and [21]).

So, optical measurements yielding information on local changes in the bilayer parameters showed that BLM thickness (except for decane BLM) decreases (if at all) by no more than fractions of a percent even at prolonged voltage action ($\sim$10 min).

Thus, this result confirmed validity of the doubts expressed by some authors [20, 25–27] concerning correctness of determining the real bilayer area when obtaining potential dependence $C_M(V)$ yielding $\Delta C_M/C_M$ of about several percent. As already mentioned, two possible sources of errors are considered in the literature: (i) uncontrolled contribution of the invisible microlenses to the changed bilayer area and (ii) inadequate accuracy in determining the bilayer-annulus boundary and (or) its shape. To estimate the contribution of microlenses, Wobschall [27] calculated that with $V=0.1$ V the bilayer area would increase by 1% if the lenses constitute from 10 to 50% of the total BLM area. Such a situation, as shown above, could take place only in membranes from oxidized cholesterol+n-decane. The quantity of microlenses is ap-

preciably less in BLM from other lipids, even with decane. Therefore, we agree with Requena *et al.* [20] who think that the lenses do not introduce any remarkable error to the value of the bilayer area measured.

As to accuracy in determining the bilayer-annulus boundary position, observation (photographing) in reflected light[1] allows us to reach sufficient accuracy up to 1 μm [20]. It is different from the definition of the true form of this boundary, which substantially differs from a circle even in the case of a membrane horizontally placed in a round aperture (*private report* of Yu.G. Rovin, photograph in [20]), and all the more so in the case of a vertically arranged membrane. The form of this contour seems to depend on the annulus width and the value of the contact angle and thus on voltage: the narrower the annulus and the greater the contact angle, the more the boundary contour approaches a circle. The basic error in determining S_m is apparently due to the fact that the boundary contour change is not taken into account. This explanation is rather probable since the voltage-induced S_m changes are observed only with membranes having an annulus. With membranes obtained by monolayer technique, such a phenomenon was not observed [5] even at 0.3 V, and the hexadecane BLM without visible annulus discussed above changes its capacity only by 0.2% at 0.1 V.

References

1. Andrews, D., Haydon, D.A. 1968. Electron microscope studies of thin lipid bilayer membranes. *J. Mol. Biol.* **32**:149
2. Andrews, D.M., Manev, E.D., Haydon, D.A. 1970. Composition and energy relationships for some thin lipid films, and the chain conformation in monolayers at liquid-liquid interfaces. *Spec. Discuss. Faraday Soc.* **1**:46
3. Babakov, A.V., Ermishkin, L.N., Liberman, E.A. 1966. Influence of electric field on the capacity of phospholipid membranes. *Nature (London)* **210**:953
4. Bamberg, E., Benz, R. 1976. Voltage-induced thickness changes of lipid bilayer membranes and the effect of an electric field on gramicidin A channel formation. *Biochim. Biophys. Acta* **426**:570

1 The method of photographing in transmitted light introduced by White [24–26] does not permit us to detect the bilayer-annulus boundary at all. It only reveals the boundary between light and dark regions of the annulus which plays here the role of a biconcave lens. The peripheral region of annulus looks dark since it refracts (like a prism) beams so much that they do not meet the microscope objective (*see* Fig. 1*b*). When observed with a microscope of a 6° angular aperture (ordinary stereomicroscope), this boundary is located where the annulus surface deflects from the bilayer plane by an angle of 20°. We did not observed the annulus image in transmitted light in a chamber with a thin partition where such an angle cannot be reached. The author of the method proceeded from an erroneous assumption that the annulus, as distinct from the bilayer, reflects all the incident light. As seen from $r^2(\theta_1)$ curves (Fig. 1*a*), even at $\theta_1 = 60°$ each of the two annulus surfaces reflects no more than 2% of the incident light.

5. Benz, R., Fröhlich, O., Läuger, P., Montal, M. 1975. Electrical capacity of black lipid films and of lipid bilayers made from monolayers. *Biochim. Biophys. Acta* **394**:323
6. Benz, R., Janko, K. 1976. Voltage-induced capacitance relaxation of lipid bilayer membranes. Effects of membrane composition. *Biochim. Biophys. Acta* **455**:721
7. Berestovsky, G.N. 1976. Interpretation of the results of membrane investigation in transmitted polarized light. *Biofizika* **21**:389
8. Berestovsky, G.N., Gyulkhandanyan, M.Z. 1976. Contact interactions of bilayer lipid membranes. *Stud. Biophys.* **56**:19
9. Berestovsky, G.N., Frank, G.M., Liberman, E.A., Lunevsky, V.Z., Razhin, V.D. 1970. Electrooptical phenomena in bimolecular phospholipid membranes. *Biochim. Biophys. Acta* **219**:263
10. Berestovsky, G.N., Lunevsky, V.Z. 1971. Electrooptical effects in membranes. *In:* Biofizika Membran. Part I, p. 111. Kaunas, USSR, (1974. *Rev. Roum. Physiol.* **11**:153)
11. Berestovsky, G.N., Lunevsky, V.Z. 1974. Measurement of membrane thickness, main refractive indexes and their small changes. The theory of method. *In:* Biophysics of Living Cell. Vol. 5 (Membranes). G.M. Frank, chief editor. p. 109. Institute of Biolog. Fiziki Akad. Nauk SSSR, Pushchino, Moscow region
12. Bunce, A.S., Hider, R.C. 1974. The composition of black lipid membranes formed from egg-yolk lecithin, cholesterol and *n*-decane. *Biochim. Biophys. Acta* **363**:423
13. Charry, R.I., Chapman, D. 1969. Optical properties of black lecithin films. *J. Mol. Biol.* **40**:19
14. Cohen, L.B. 1973. Changes in neuron structure during action potential propagation and synaptic transmission. *Physiol. Rev.* **53**:373
15. Elkin, A.P., Berestovsky, G.N., Gyulkhandanyan, M.Z. 1974. The Study of influence of mono- and divalent cation concentration on the distance between two lipid bilayers in the contact zone. *Dokl. Akad. Nauk SSSR* **218**:1454
16. Fettiplace, R., Andrews, D.W., Haydon, D.A. 1971. The thickness, composition and structure of some lipid bilayers and natural membranes. *J. Membrane Biol.* **5**:277
17. Kruglyakov, P.M., Rovin, Yu.G., Bagaveev, L.A., Koretsky, A.F. 1972. Black hydrocarbon films in aqueous mediums. III. Influence of organic phase nature on the thickness and molecular interaction. *Izv. Sib. Otd. Acad. Nauk SSSR. Ser. Khim. Nauk* **6**:(N3):12
18. Pasechnik, V.I., Sokolov, V.S. 1973. Permeability change of modified bimolecular phospholipid membranes accompanying periodical expansion. *Biofizika* **18**:655
19. Requena, J., Haydon, D.A. 1975. The Lippmann Equation and the characterization of black lipid films. *J. Colloid Interface Sci.* **51**:315
20. Requena, J., Haydon, D.A., Hladky, S.B. 1975. Lenses and the compression of black lipid membranes by an electric field. *Biophys. J.* **15**:77
21. Rosen, D., Sutton, A.M. 1968. The effects of a direct current potential bias on the electrical properties of bimolecular lipid membranes. *Biochim. Biophys. Acta* **163**:226
22. Rovin, Yu.G. 1976. Changes of the tension of black hydrocarbon film in aqueous medium under the action of potential difference. *Kolloid Zh.* **38**:215
23. Vol'kenshtein, M.V. 1951. Moleculyarnaya Optica. p. 744. Gos. Izdat., Moscow
24. White, S.H. 1970. Thickness changes in lipid bilayer membranes. *Biochim. Biophys. Acta* **196**:354
25. White, S.H. 1970. A study of lipid bilayer membrane stability using precise measurements of specific capacitance. *Biophys. J.* **10**:1127
26. White, S.H., Thompson, T.E. 1973. Capacitance, area and thickness in thin lipid films. *Biochim. Biophys. Acta* **323**:7
27. Wobschall, D. 1972. Voltage dependence of bilayer membrane capacitance. *J. Colloid Interface Sci.* **40**:417