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STUDIES OF EXCITABLE MEMBRANE FORMED ON THE SURFACE OF PROTOPLASMIC DROPS ISOLATED FROM *NITELLA*

III. IMPEDANCE OF THE SURFACE MEMBRANE

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SUMMARY

A protoplasmic drop isolated from an internodal cell of *Nitella* in an initial solution composed of 70 mM KNO_3, 50 mM $NaNO_3$ and 5 mM $CaCl_2$ became electrically excitable when the drop was placed in the final solution containing 0.5 mM KNO_3, 0.5 mM NaCl, 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$. The electrical impedance of the surface membrane of the drop was measured both in the initial and final solutions at frequencies between 60 Hz and 100 kHz.

The impedance and admittance loci of the surface membrane fell on circular arcs. The d.c. resistance $R_m°$, and the d.c. capacitance $C_m°$ were determined by extrapolating the circular arcs to the low frequency limit. $R_m°$ thus determined was in the range of 50–200 $\Omega \cdot cm^2$ in the initial solution, and increased to a steady value of 0.4–4.0 $k\Omega \cdot cm^2$ when the external solution was replaced by the final solution. After the protoplasmic drop was isolated from the internodal cell of *Nitella*, $C_m°$ decreased monotonically from about 1.5 $\mu F/cm^2$ within 20 min and approached 1.25 ± 0.1 $\mu F/cm^2$ both in the initial and final solutions. No appreciable difference was observed for $C_m°$ in these two solutions.

The impedance data were discussed in relation to the process of formation of the membrane at the surface of the protoplasmic drop. After the excitable stage was reached, the drop membrane impedance was found to decrease by a factor of 10 during excitation.

INTRODUCTION

When an internodal cell of *Nitella* is amputated near the end of the cell in a solution containing 70 mM KNO_3, 50 mM $NaNO_3$ and 5 mM $CaCl_2$, the protoplasm effuses from the opening of the cell, and forms a sessile drop on the bottom of the vessel[1]. A thin membrane of less than 100 Å in thickness is formed at the surface of the drop[2,3]. This protoplasmic drop membrane becomes electrically excitable when the external solution is changed to an appropriate salt solution, *i.e.* 0.5 mM KNO_3, 0.5 mM NaCl, 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$[3,4]. For convenience we refer to these solutions as the "initial" and "final" solutions, respectively, in this article. The excitable membrane of the protoplasmic drop is suitable

for studying the structure of the membrane and the molecular mechanism of various functions of the membrane. Based on the experimental results thus far obtained for the membrane potential, electric resistance, refractive index and surface tension of the membrane, we have inferred that the process of formation of the excitable membrane on the drop surface is accompanied by an appropriate conformation of the phospholipids and proteins which have come out from the inside of the protoplasmic drop[5,6].

In this study, we have measured the impedance of the drop membrane by using an appropriate glass capillary[7] for obtaining more accurate information about the process of formation and structure of the membrane. The static capacitance and conductance of the drop membrane both in the initial and the final solutions, and its time-course in the formative period of the functional membrane are measured in the wide range of frequencies applied.

EXPERIMENTAL

Materials

The *Nitella sp.* used in this study was the same as that used in Parts I and II of this series[5,6]. The protoplasm was isolated in the initial solution mentioned above from the internodal cell of *Nitella* by the method employed by Kamiya[1]. The external solution was then replaced by the excitability testing of the final solution. The osmolarity of these solutions was adjusted by adding recrystallized mannitol, and the pH of the final solution was adjusted to about 7.0 by Tris–acetate buffer.

All measurements were performed in an air oven regulated to 20 ± 2 °C.

Measurement of membrane impedance

The impedance of the surface membrane of the protoplasmic drop was measured by use of a capillary as illustrated schematically in Fig. 1. Details of the top portion of the capillary are shown in Fig. 1b and 1c. The capillary was made as follows. One end of a clean thin-wall glass capillary of 1 or 1.5 mm in diameter was heated until the opening became 150 or 200 μm in diameter. The opposite end of the capillary was connected to a microsyringe by polyethylene tubing. Two platinized platinum electrodes coated with an insulating enamel except at the tip portion were inserted into the glass capillary as shown in Fig. 1. The one electrode was used for the impedance measurement and the other for electric stimulation of the drop membrane.

The impedance of the surface membrane of the drop was measured by a dielectric loss bridge (Ando Electric Co. Ltd, Type TR-1C) combined with a G-C box and an oscillator. The output of the bridge was connected to a tuned null detector through a differential preamplifier. The applied frequency examined was between 60 Hz and 100 kHz. The details of the apparatus of the impedance measurement were essentially the same as those used in our previous studies[8,9]. The output of the null detector was monitored with an oscilloscope.

A large capacity C (1.0 μF) was inserted in series as shown in Fig. 1a to prevent d.c. current in the bridge circuit. In the measurement of the impedance of the drop membrane, the a.c. voltage applied through a pair of Pt–Pt electrodes across the drop

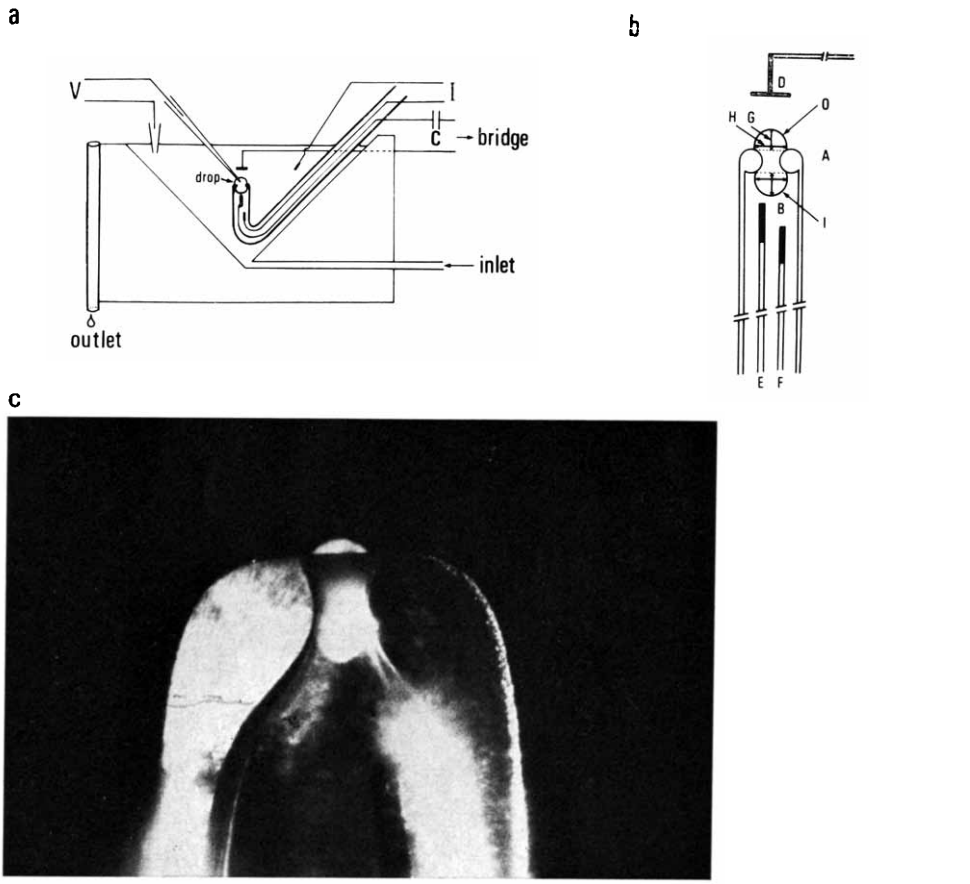

Fig. 1. (a) Schematic diagram of the lucite vessel and the glass capillary cell used for measuring the electrical response and impedance of the membrane. Transmembrane potential was recorded with a microelectrode inserted into the drop. The reference electrode (saturated calomel electrode) was connected with the external solution through KCl salt bridge. Two pairs of electrodes, one for current stimulation and the other for impedance measurements were Pt–Pt electrodes. C was the capacity (1 μF) to prevent the d.c. in the bridge circuit. (b) The top portion of the capillary. A, external solution; B, internal solution; D and E, external (D) and internal (E) Pt–Pt electrodes for impedance measurement. F, internal Pt–Pt electrode for current stimuli. I and O, inner (I) and outer (O) portions of the surface membrane of the drop in the capillary. (c) Photograph of the drop in the top of the capillary.

was maintained in the range from less than a few mV to prevent disturbance of the functional membrane.

A protoplasmic drop was isolated from an internodal cell, and was placed on the top of the opening of the capillary. Then the drop was sucked into the capillary as shown in Fig. 1. This procedure was performed by handling a microsyringe by means of a micro-manipulator. The drop in the capillary did not change its shape during the impedance measurements. The surface area of the drop was evaluated by measuring the sizes (G and H in Fig. 1b) of the outer and inner portions of the drop with a microscope. The error involved in the surface area thus determined was smaller than $\pm 5\%$. In the studies described in this article, the area of the outer or inner

portion of the surface membrane ranged from $0.4 \cdot 10^{-3}$ cm^2 to $2.0 \cdot 10^{-3}$ cm^2 depending on the size of the drop used.

Measurement of transmembrane potential

A glass microelectrode filled with 3 M KCl solution was inserted into the drop to measure the transmembrane potential (see Fig. 1a). The reference electrode was a saturated calomel electrode connecting electrically with the external solution through a 0.3 M KCl salt bridge. In order to prevent the contamination of the external solution caused by KCl leaking from the salt bridge, the bridge was placed as close to the outlet of the lucite vessel as possible. The potential was monitored by an oscilloscope through a high input impedance d.c. preamplifier. Electric current stimuli were delivered between the two platinized platinum electrodes from an electric stimulator (Nihon Koden, Type MSE-3R) through a series resistor of 20 MΩ.

RESULTS AND DISCUSSION

Frequency dependence of membrane capacitance C_m and conductance G_m

In order to measure the membrane impedance accurately, it is necessary to reduce the impedance of the external solution compared with that of the membrane impedance. It was possible to reduce the electric resistance between two electrodes so that it was much less than that of the surface membrane by putting the inner electrode (E) close to the inner surface (I) of the drop (see Fig. 1b). The resistance of the solution in the outer compartment (A) was negligibly small in comparison with that in the capillary (B) (see Fig. 1b). Actually the measured resistance was independent of the position of the external electrode (D). The observed impedance of the membrane was represented in terms of a parallel circuit, *i.e.* a capacitance c_p in parallel with a conductance g_p. The observed value of the parallel resistance, $1/g_p$, was the order of 1 MΩ in the low frequency region for every protoplasmic drop in the final solution, while the electric resistance of the final solution in the capillary was evaluated to be about 40 kΩ/mm. This implies that a sufficiently accurate measurement of the membrane impedance can be made by using the cell arrangement given in Fig. 1a provided that the inner electrode (E in Fig. 1b) is placed as close to the membrane as possible.

The impedance of the surface membrane was measured both in the initial and the final solutions. As the measurements were performed in the present study under the condition that the two surface membranes of the drop, O and I in Fig. 1b, were placed in series between a pair of electrodes, the observed values of c_p and g_p could be reduced to the values of C_m and G_m per unit area of the membrane by using the following equations.

$$\frac{1}{c_p} = \frac{1}{S_0 C_m} + \frac{1}{S_i C_m}$$

$$\frac{1}{g_p} = \frac{1}{S_0 G_m} + \frac{1}{S_0 G_m}$$

where S_0 and S_i stand for the area of the two portions O and I of the surface membrane of the drop in the capillary (see Fig. 1b). Here the capacitance and conductance

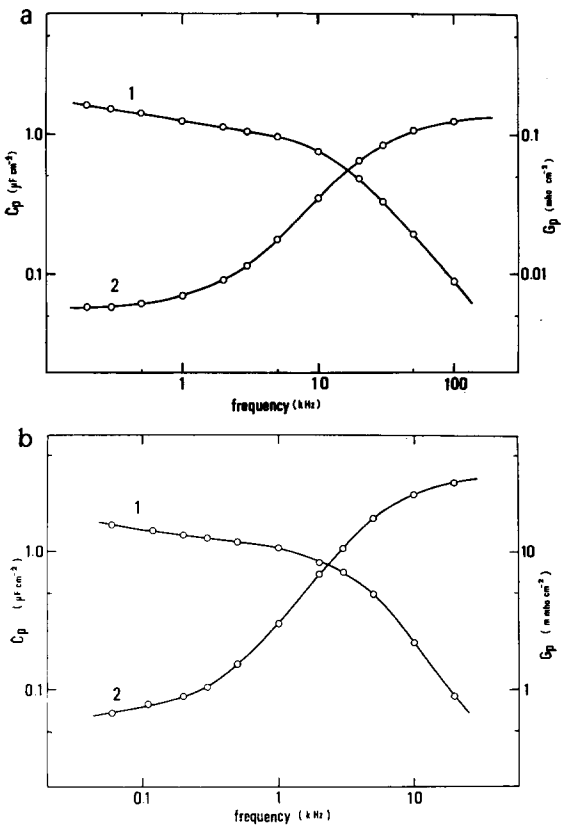

Fig. 2. Typical data of C_m (line-1) and G_m (line-2) as functions of applied frequency, f, in the initial solution (a) and the final solution (b).

of the inner and outer membranes are assumed to have the same value per unit area.

Figs 2a and 2b show the values of C_m and G_m thus obtained in the initial and the final solutions as functions of applied frequency f, where both the ordinate and abscissa are written in logarithmic scales. The values of C_m remain constant in the low frequency region, while they decrease rapidly with increase of frequency. On the other hand, the values of G_m become constant both in high and low frequency extremes. The frequency dependencies of C_m and G_m in the final solution are similar to those in the initial solution, except that the characteristic frequency of dispersion of C_m in the initial solution is appreciably higher than that observed in the final solution.

Impedance and admittance loci of the surface membrane of the protoplasmic drop

The values of C_m and G_m can be transformed into the sequence of a resistance, R_s and a reactance, X_s. The values of R_s and X_s are calculated from C_m and G_m according to the following equations

$$R_s = 1/G_m(1+\omega^2 C_m^2 G_m^{-2})$$

$$X_s = -\omega C_m/G_m(1+\omega^2 C_m^2 G_m^{-2})$$

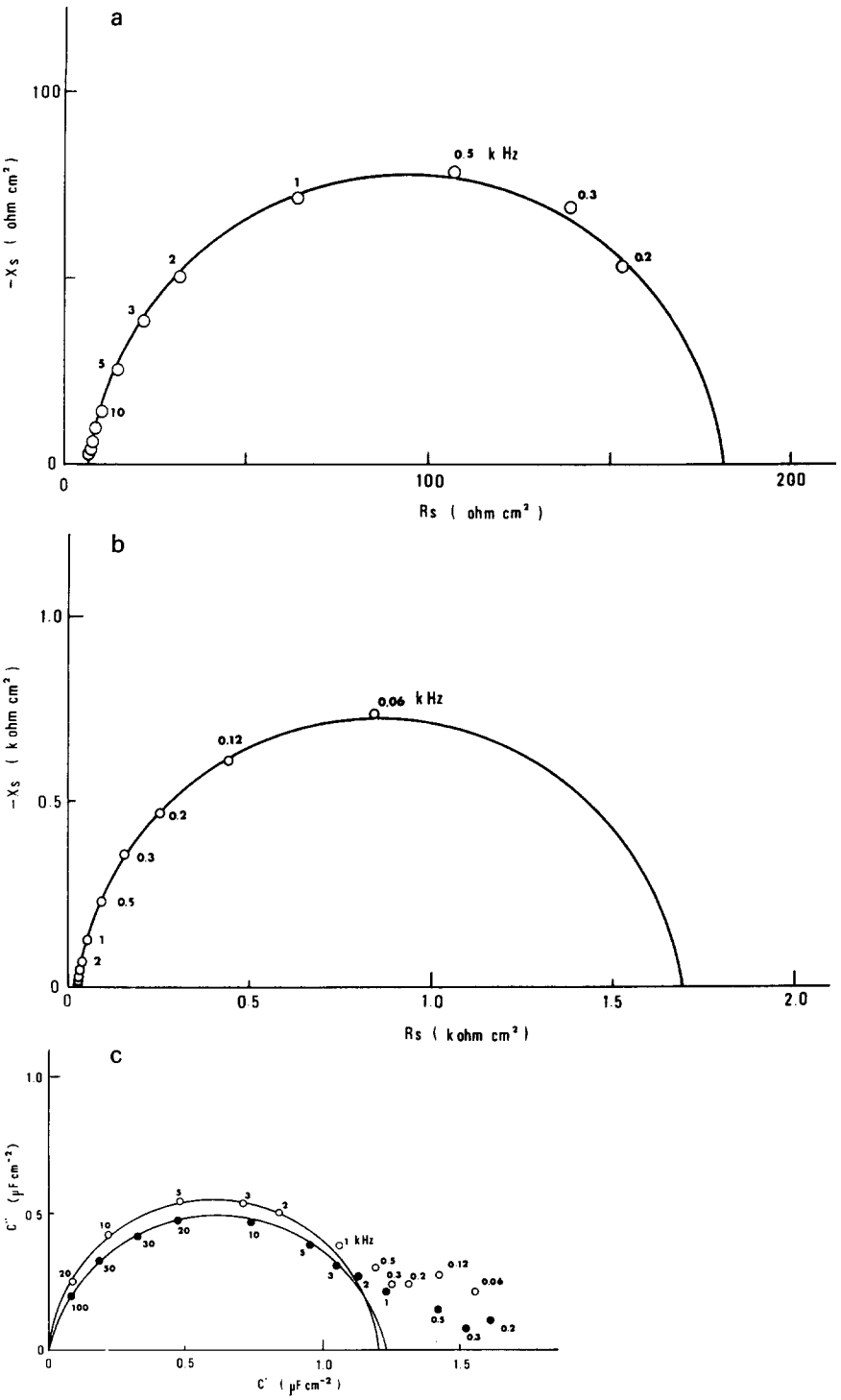

Fig. 3. Impedance and admittance loci of the surface membrane. The drop studied in the initial solution (a) and the final solution (b) are the same as with those in Fig. 2. In Fig. (c). ○—○, data in the final solution, and ●—●, data in the initial solution.

where ω is the angular frequency defined by $\omega = 2\pi f$. Fig. 3a shows the impedance locus of the surface membrane of the drop in the initial solution, and Fig. 3b shows this in the final solution. As seen in Figs 3a and 3b, the impedance loci of the surface membrane give circular arcs both in the initial and the final solutions. The d.c. resistance of the surface membrane, R_m°, is obtained from the intercept of the circular arc on the abscissa by extrapolating to the low frequency extreme. The value of R_m° thus obtained was 180 $\Omega \cdot cm^2$ for the drop in the initial solution (Fig. 3a), while in the final solution R_m° increased to 1.7 $k\Omega \cdot cm^2$ (Fig. 3b). The other drop specimens gave the same order of magnitude for the electric resistance of the surface membrane as shown in Table I.

TABLE I

The values of C_m° and R_m° obtained from drops in the initial and the final solutions.

Drop No.	*Environmental solution*	C_m° ($\mu F/cm^2$)	R_m° ($\Omega \cdot cm^2$)
20	initial solution	1.33	55
21		1.25	60
24		1.31	90*
		1.29	140
29		1.23	180
25	final solution	1.18	410
26		1.30	450
30		1.20	466
31		1.32	730
50		1.17	1400
56		1.15	1500
58		1.21	3700
62		1.27	470
63		1.20	1700

* This value was obtained at the formative period in the initial solution.

To obtain the d.c. membrane capacitance C_m°, the impedance data are replotted in the admittance plane, where $(G_m - G_m^\circ)/\omega$ are plotted against C_m. The admittance loci of the drop in the initial and the final solutions are shown in Fig. 3c. The open circles show the data in the initial solution, and the closed circles are those in the final solution. The data fall on circular arcs in a high frequency region and deviate from the arc with decrease in frequency. The intercept of the extrapolated circular arc at the C_m axis gives the d.c. capacitance C_m° of the membrane. C_m° thus obtained is 1.23 $\mu F/cm^2$ in the initial solution, and 1.20 $\mu F/cm^2$ in the final solution. The center of the admittance locus lies slightly under the real (C_m) axis in all cases studied here.

The results of impedance measurements of the surface membranes of various protoplasmic drops examined in the two types of solutions are summarized in Table I. The values of R_m° are less than 0.2 $k\Omega \cdot cm^2$ in the initial solution, and vary between 0.4 and 4.0 $k\Omega \cdot cm^2$ in the final solution. These values agree well with those obtained from the I–V relation reported in Part I[5]. On the other hand, no significant difference was detected among the values of C_m° in the initial and the final solutions. The obtained value of C_m° was 1.25 ± 0.1 $\mu F/cm^2$ for all drops studied.

Variation of the membrane impedance with time during the formative period of the surface membrane

Protoplasmic drops were not electrically excitable in the initial solution. It required about 1 h for the drop to become electrically excitable after the replacement of the external medium by the final solution. The time-course of variation of the membrane potential, the d.c. resistance, the refractive index and the tension of the surface membrane during the formative period of the functional membrane of the drop in the final solution were discussed in detail in Parts I and II of this series[5,6]. It was suggested that the surface membrane of the drop is formed initially by phospholipids, and then proteins in the protoplasm are penetrating through the surface membrane, which leads to an appropriate molecular conformation responsible for the occurrence of excitability of the drop membrane. Although the capacitances of the membrane in the initial and the final solutions have no appreciable difference with each other, we can check the process of formation of the surface membrane described above by the impedance measurements.

A protoplasmic drop was isolated in the initial solution on the top of the capillary and sucked into the capillary. Immediately after the drop was settled into the capillary, the external solution was switched from the initial solution to the final one as fast as possible and the impedance was measured. In Fig. 4a the relative membrane capacitance $C_m°/(C_m°)_s$ is plotted against the time after the replacement of the external

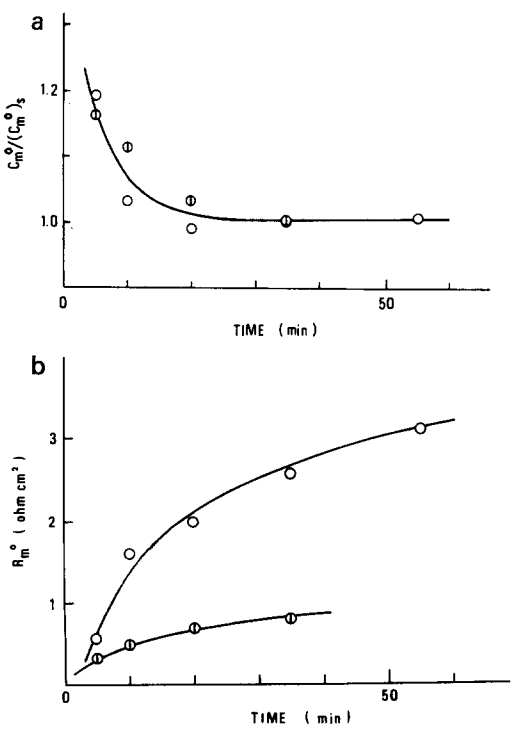

Fig. 4. Time-courses of membrane capacitance (a) and of membrane resistance (b) of protoplasmic drops during formative processes of the surface membrane in the final solution. Different symbols refer to different drop specimens.

solution, where $(C_m°)_s$ refers to the membrane capacitance in the steady state. Two examples of the time-course of the membrane resistance, $R_m°$, are shown in Fig. 4b. Note that the value of $R_m°$ differs from one drop to the other (see Table I). It is seen in the figure that $C_m°$ decreases and approaches the steady value within 20 min while $R_m°$ continues to increase for more than 50 min. However, no difference in the values of $C_m°$ was observed by replacement of the external medium with the final solution after $C_m°$ had attained the steady value in the initial solution. Note that the membrane resistance, $R_m°$, was entirely different in these two solutions as seen in Table I.

For comparative purposes, the time-courses of the refractive index, surface tension, membrane resistance are compared with that of the membrane capacitance in the final solution in Fig. 5. The drop became electrically excitable in response to an external stimulus about one hour after the medium was replaced by the final solution. In the figure, the excitable range was shown by region B–C, *i.e.* the right side of the dotted vertical line. All of these physicochemical properties of the surface membrane except the membrane capacitance $C_m°$ changed in parallel in the formative processes (Region A–B in Fig. 5). It is noted that the variation of $C_m°$ proceeded much faster than that of the other properties. This implies that the change of $C_m°$ with time reflects a different molecular mechanism in the formative period of the surface membrane from those obtained from the other properties. As pointed out above, the time-course of variation of $C_m°$ in the final solution was almost the same as in the initial solution. Therefore, it may not be unreasonable to consider that the decrease in $C_m°$ stems mainly from the variation of membrane thickness during the formation of the phospholipid surface membrane. However, the permeability of ions across the surface membrane in the initial solution has been observed to be extremely high for all univalent cation species compared with those in the final solution (Ishida, N., Inoue, I. and Kobatake, Y., in preparation). Considering the various experimental facts described above, together with the fact that the surface membrane formed in the initial solution gave almost the same value of the refractive index as that with the phospholipids[5], we may infer that the membrane formed at the surface of the drop is not a compact lipid bilayer membrane; instead, the membrane is considered

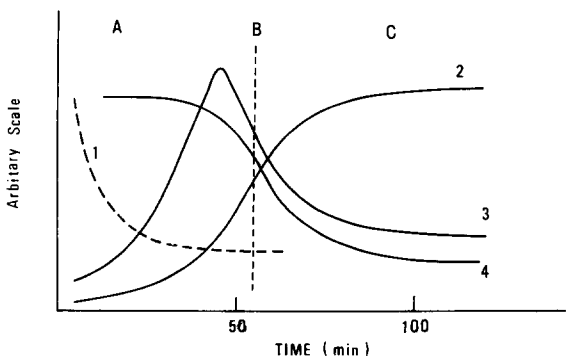

Fig. 5. Diagramatical comparison of time-courses of physicochemical properties of the surface membrane of drops during its formative processes in the final solution. Lines numbered 1, 2, 3 and 4 show the time-course of the membrane capacitance, surface tension, membrane resistance, and refractive index, respectively.

to be an aggregate of micelles of phospholipids. The penetration of the proteins which is responsible for the occurrence of the excitability of the surface membrane does not lead to an appreciable change in the membrane capacitance. However, the membrane resistance, membrane potential and the surface tension change with time during this penetration process[6].

Variation of the membrane impedance during excitation

Protoplasmic drop membrane in the final solution is electrically excitable in response to external stimulus. However, we have not mentioned that the drop membrane retains its excitability even in the capillary employed in the present study. When we apply an outwardly directed electric current, the membrane suffers a hyperpolarizing current stimulus which does not induce excitation for the inside membrane. On the other hand, the outside membrane is depolarized by the applied current and excitation is induced when the current strength exceeds the threshold value. This process of excitation can be observed by measuring the membrane potential between the microelectrode inserted in the drop (Fig. 1a) and the reference electrode. The upper traces in Fig. 6 show the variations of the transmembrane potential when a rectangular electric current of a given strength is passed through the drop. Figs 6a and 6b refer the cases where the current strength is fixed at 25 μA/cm^2 and 30 μA/cm^2, respectively. The drop used here had the resting potential of -55 mV, the d.c. resistance of about 2 k$\Omega\cdot$cm^2 and the threshold value of stimulating current strength of 20 μA/cm^2. The lower traces in Fig. 6 show the unbalance of the impedance bridge during excitation. It is noted that the unbalance of the bridge is not observed when the membrane is in the subthreshold state, while in the excited state the impedance decreases by more than a factor of 10. The bridge was balanced at the resting state with an applied frequency of 1 kHz. As pointed out above, the electric capacitance was not changed appreciably both in the initial and the final solutions. Therefore,

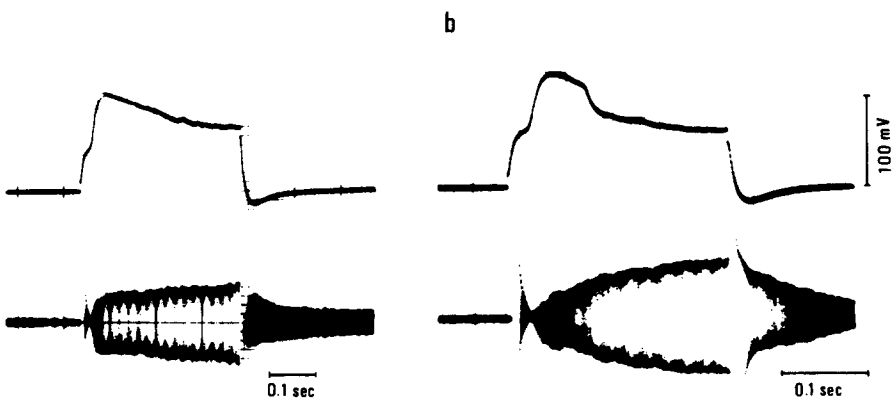

Fig. 6. The changes of transmembrane potential and membrane impedance during excitation. Upper traces indicate the transmembrane potential, and lower traces indicate the change of membrane impedance. The bridge was balanced at the resting state with 1 kHz in frequency. The resting potential of the drop was -55 mV and the d.c. resistance was about 2 k$\Omega\cdot$cm^2. Current strength applied was fixed at 25 μA/cm^2 for (a) and 30 μA/cm^2 for (b). The threshold value of stimulating current strength was 20 μA/cm^2 for this specific drop.

the unbalance of the impedance bridge during the process of excitation stems from a variation of the electric conductance of the membrane. This is the same as that observed in an ordinary nervous tissue[10–14].

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