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A new system for bilayer lipid membrane capacitance measurements: method, apparatus and applications

Sławomir Kalinowski and Zbigniew Figaszewski

Institute of Chemistry, University of Warsaw, Białystok Branch, Białystok (Poland)

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A new method and a new apparatus for capacitance measurements on bilayer lipid membranes are described. The membrane is charged and discharged with a constant current during the measurement. The charge-discharge cycle duration, which is proportional to the membrane capacitance, is measured. The measured time period is converted into a binary number by digital systems and then this number is either further converted into a constant capacity-proportional voltage or read out by the computer. The apparatus makes it possible to measure the capacitances of voltage-polarized membranes. Application of the apparatus to capacitance measurements of bilayer lipid membranes during their potential on the capacitance is presented. The capacitances of membranes stimulated by rectangular voltage pulses and of those stimulated by a linearly varying potential were reported.

Introduction

The cell built of plasma and surrounded by the membrane layer is an essential element of all living organisms. The studies on cell membranes have provided evidence that the main membrane-forming structure is the lipid bilayer. Formation of a synthetic planar bilayer lipid membrane (BLM) made it possible to extend the investigation methods used in the studies of membrane phenomena. A number of electroanalysis techniques have become available when it became possible to place the electrodes on both membrane sides [1–6]. Numerous investigators are nowadays studying various topics of cell membrane functions. Some control and transport mechanisms of cell membranes were reproduced in synthetic membranes [7–9]. This permitted to study the cell membrane functions and the associated phenomena in detail.

Synthetic lipid bilayers can be formed as liposomes, where the lipid bilayer surrounds an electrolyte droplet [10]. The experimental methods used for those systems are similar to those applied on studies on natural cell membranes.

Lipid bilayers can also be formed as planar membranes spread over a hole in a hydrophobic wall separating two electrolyte solutions [1–5]. As a rule, the hole diameter is from 0.5 to 3 mm. On such an arrangement many electroanalytical techniques could be applied which are not practicable on native cells and liposomes.

Planar bilayer lipid membranes could be formed by two methods. One of them is based on the Langmuir-Blodgett technique. It consists in formation of a bilayer by superposition of two monolayers [1,5]. The other method, a more simple one, is the membrane formation from a lipid solution. A drop of such a solution is placed in the hole of the septum. The solvent and the lipid excess pass to the solution and the lipid bilayer is formed in the hole after a time period [1,2,5]. Decane and other hydrocarbons are usually used as solvents. Admixtures of other solvents, e.g., chloroform or butanol, are also used.

The membrane formation stage can be estimated optically by its observation in the reflected light [11,12]. Disappearence of light reflection is observed when the membrane attains the lipid bilayer form due to very low membrane thickness (4–13 nm). Such membranes are also called 'black membranes'.

More information on the membrane formation process can be obtained from the capacitance measurements. The characteristic capacitance of bilayer membranes is 0.3–1.3 $\mu F/cm^2$ [5].

Combining the capacitance measurement with the bilayer area measurement in reflected light makes it possible to determine the specific capacitance, charac-

Correspondence to: Z. Figaszewski, Institute of Chemistry, University of Warsaw, Białystok Branch, Al. J. Piłsudskiego 11/4, 15-443 Białystok, Poland.

teristic for the properties of the bilayer part of the membrane [1].

The membrane capacitance measurements are usually carried out by bridge methods. Low-voltage bridges are used in such methods which stimulate the membrane with a voltage of 10–30 mV amplitude [1,12–14]. The membrane capacitance can be determined from the membrane capacitance-current measurement applying the alternate voltage [1]. The membrane capacitance can also be determined by the mathematical analysis of the membrane response to a weak voltage or current pulse [14–16].

These methods do not allow a simple and rapid capacitance measurement of the membranes. The capacitance measurements of a membrane with applied membrane potential are particularly troublesome These complications have prompted us to elaborate and to manufacture an instrument which would enable one to read out and to record the membrane capacitance continuously and to measure the capacitance of membranes polarized by a voltage.

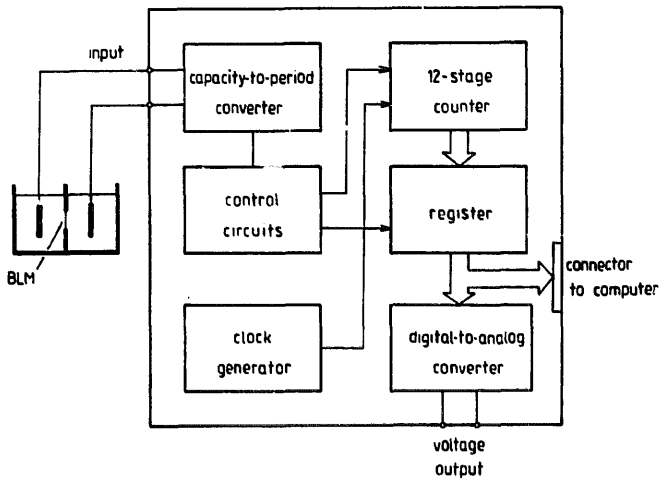


Fig. 1. Block diagram of the capacity-to-voltage converter.

Description of the instrument

Operation principle

The instrument converted the membrane capacitance by an analog-digital procedure. The block diagram of the instrument is presented in Fig. 1. The essential unit of the instrument is the capacitance converter to rectangular pulses of period proportional to the input capacitance. The clock generator pulses are counted up by a 12-bit counter. A counting time is determined by the pulse period on the output of the capacity-to-period converter. The control circuits control the start and the end of the clock pulses counting and generate the pulses making the counter content by written down by the register and the counter be set zero. The register content is transmitted to the 12-bit digital-to-analog converter. Its output voltage is proportional to the 12-bit binary number on its inputs, i.e., to the input capacitance. The register content can also be read out by a computer.

Capacity-to-period converter

The capacity-to-period converter is the essential element of the instrument. In determines the characteristics of the whole instrument. Its diagram is presented in Fig. 2.

Two capacities, C_1 and C_X, are shown in the figure. C_1 is the capacitor inside the converter which limits the converter response frequency at low membrane capacitance, C_X.

In a typical application of the circuit, the pin 1 (GND) is connected with the ground. In ours it is connected with the negative supply voltage. Such a modification allows to change the current direction to charge the membrane.

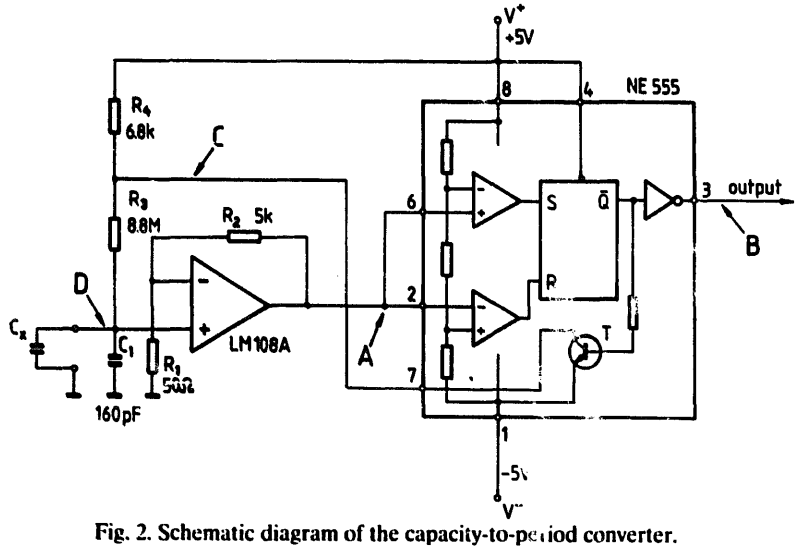


Fig. 2. Schematic diagram of the capacity-to-period converter.

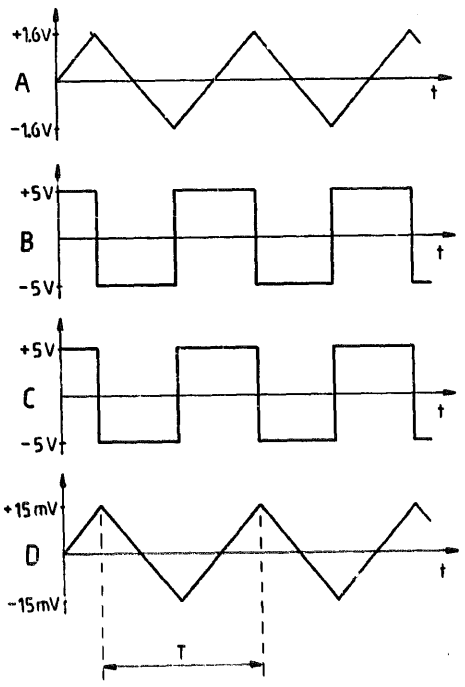


Fig. 3. Voltage wave of the capacity-to-period converter.

The points A–D are marked in Fig. 2. The voltage waveforms in these points are presented in Fig. 3.

When there is a positive potential at the pin 3 (OUTPUT, the point B) of the NE555 circuit the inner discharge transistor is open and the C_X and C_1 capacitors are charged by the R_3 and R_4 resistors (the point D). The capacitors voltage increases. It is amplified by the operational amplifier LM108A used as a non-inverting amplifier circuit. When the voltage of the pin 6 of the NE555 circuit (THRESHOLD, the point A) attains the $1/3V^+$ value then the internal RS trigger changes its state. The discharge transistor T of the NE555 circuits is switched on and connects the resistor R_3 with the negative supply voltage. The capacitances C_X and C_1 are discharged and are charged by a current with opposite direction. The voltage of the point A shifts over to negative values.

If the voltage of the pin 2 ($\overline{\text{TRIGGER}}$, the point A) attains the $1/3V^-$ value then the state of the pin 3 (the point B) changes and the discharge transistor T remains open. The input capacities C_1 and C_X are charged again. The cycle resumes. As the capacitance C_X increases, the voltage of this capacitor increases slower and the charge/discharge cycle of the input capacities becomes longer. The output pulse period is then longer.

The effect of parameter values on the performance of the capacity-to-period converter

The voltage is fed to the NE555 circuit in an untypical way in the instrument. The pin 1 of the NE555 system (GND) is connected with the negative feeding voltage. For this reason, the voltage of the pin 3 changes at the voltages $1/3V^-$ of the pin 2 and $1/3V^+$ of the pin 6.

The membrane voltage is amplified by the operational amplifier LM108A. As the voltage of the inputs 2 and 6 of the NE555 system varies within the $1/3V^-$ $1/3V^+$ limits, the membrane voltage depends on the voltage supply and on the amplification of the non-iverting amplifier with the LM108A circuit.

The amplification degree k is given by the equation:

$$k = \frac{R_1 + R_2}{R_2} \tag{1}$$

If the symmetrical voltage is fed to the system then

$$V^+ = |V^-| = V \tag{2}$$

The voltage amplitude peak-to-peak across the membrane, U_M, is given by

$$U_M = \frac{2}{3} \cdot V \cdot \frac{1}{k} = \frac{2 \cdot V \cdot R_1}{3 \cdot (R_1 + R_2)} \tag{3}$$

A compromise should be thought over when choosing the voltage amplitude across the membrane. A low amplitude value is desired because of small effects of the measurement on the membrane phenomena but it lowers the measurement accuracy because of the effect of noises of the measuring system and of the transformer.

At low voltage amplitude across the membrane is loaded by a constant current. The generator wave period can be described by the equation:

$$T = T_U + T_D = \frac{U_M \cdot (R_3 + R_4) \cdot C}{V} + \frac{U_M \cdot R_3 \cdot C}{V} \tag{4}$$

where T_U = the membrane charging time to the U_M^+ voltage, T_D = the membrane charging time to the U_M^- voltage, C = the total capacitance of the membrane, of the connecting cable and of input capacitance of the converter.

If $R_3 \gg R_4$ then the Eqn. 4 assumes a more simple form.

$$T = \frac{2 \cdot U_M \cdot R_3 \cdot C}{V} \tag{5}$$

An increase in R_3 decreases the generator frequency and hereby improves the system stability. However, the error caused by membrane conductivity increases in this case but, on the other hand, the error due to the electrolyte and electrode resistance decreases.

Fig. 4 shows the dependence of output waveform period and frequency upon the input capacitance C_X.

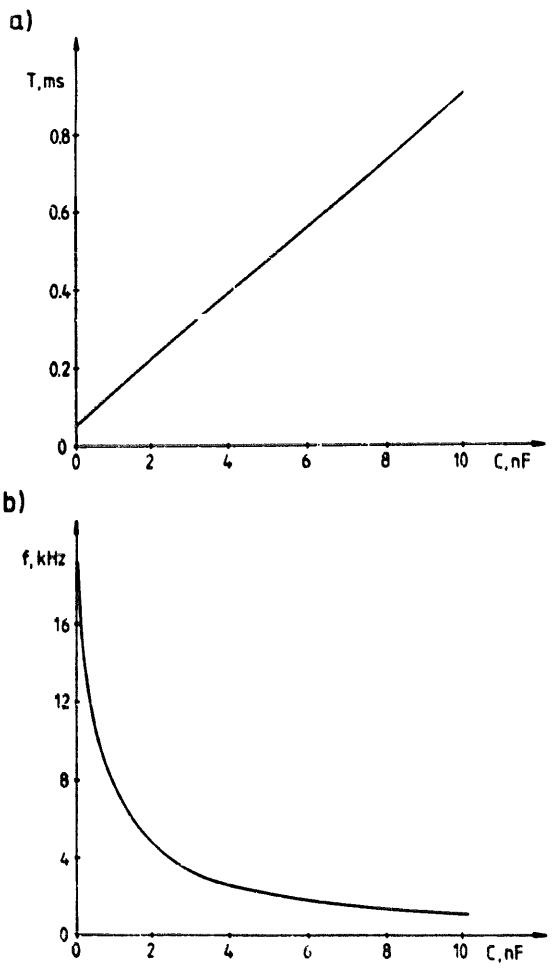


Fig. 4. Frequency (a) and period (b) of the capacity-to-period converter as the input capacitance function.

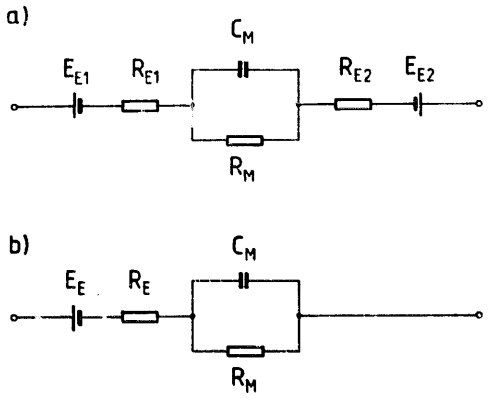
Measurement errors

The membrane, the electrolyte and the electrodes together are a system of resistance, capacitance and voltage sources (Fig. 5). In the capacitance measurements, the effects of all the individual components should be taken into account.

The membranes are characterized by high resistance which practically does not cause any capacitance measurement error. The effect of the membrane on the measurement error is shown in Fig. 6a. It can be assumed based on the studies carried out hitherto [3] that the membrane resistance is high enough and does not cause any measurement error.

The capacitance measurement accuracy is far more affected by the sum of electrode and electrolyte impedances. Their effects on the measurement error are presented in Fig. 6b.

Low-impedance electrodes and electrolytes should be used for the membrane capacitance measurements with the instrument presented here. The calomel electrodes are not suitable to this aim. The silver-silver chloride electrodes directly immersed in the electrolyte



R_M - membrane resistance

C_M - membrane capacitance

R_E, R_{E1}, R_{E2} - electrode and electrolyte impedances

E_E, E_{E1}, E_{E2} - electrode potentials

Fig. 5. Simple equivalent circuits of electrodes, electrolytes and membranes: (a) membrane separating two electrolytes, (b) membrane on solid state electrode.

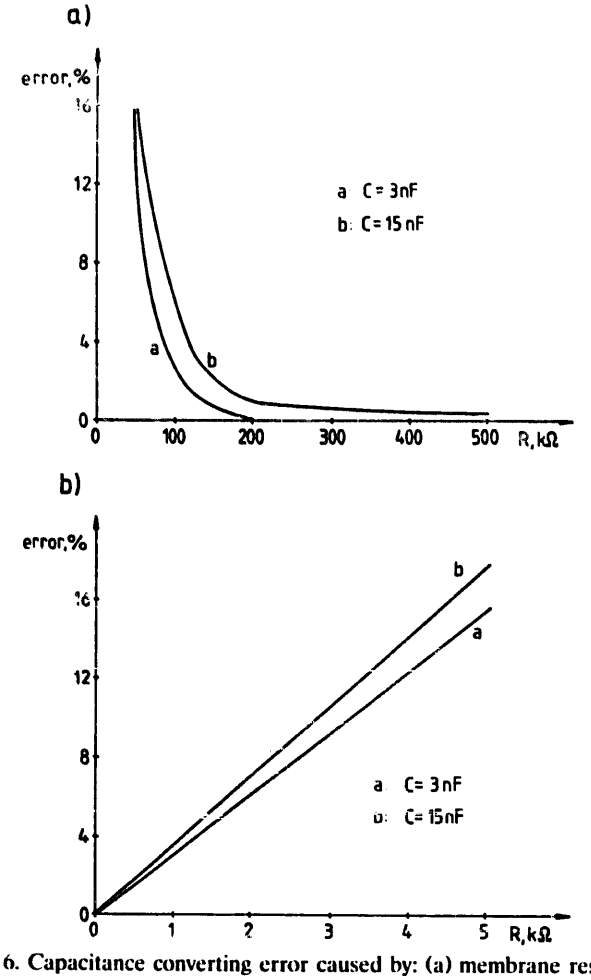


Fig. 6. Capacitance converting error caused by: (a) membrane resistance, (b) electrodes and electrolytes impedance.

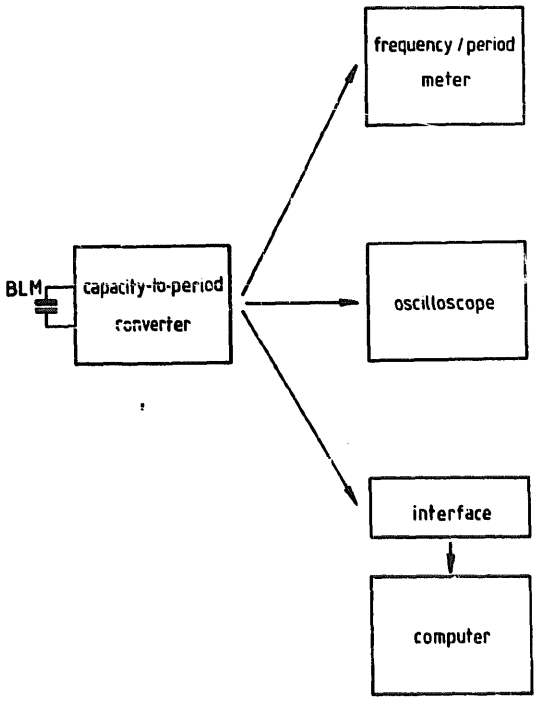


Fig. 7. Adaptation of the capacity-to-period converter for measurements of the membrane capacitance.

solution which is present on both membrane sides are the best.

A decrease in membrane resistance can result in increased measurement error when the capacitance measurements with imposed potential are carried out.

Possibilities of applying the capacity-to-period converter

The capacity-to-period converter (Fig. 2) is a very simple, inexpensive and easy to build system. It can be used for membrane capacitance measurements but it does not make it possible to record the capacitance with an *Y-t* plotter.

The way of applying the capacity-to-period converter is presented in Fig. 7. The period or the frequency of the converter terminals can be measured with a frequency/period measuring device or with an oscilloscope. The capacitance is read out from a calibration curve (Figs. 4a and 4b).

It is possible to connect the converter to a computer using various interfaces. The period or the frequency are then read out using a software procedure. It is possible to process and analyse the experimental data there after. The results can be presented graphically on the monitor, plotted or printed.

Possibilities to apply the capacity-to-voltage converter

The instrument makes it possible to measure and to record the membrane capacitance in a simple way (Fig. 8a). A capacitor plate is earthed. It is a great advantage of the instrument because one can connect, e.g., a constant voltage source, a linear sweep generator, or a rectangular pulse generator between that plate and the shunt and then measure or record the membrane capacitance with the applied membrane potential (Fig. 8b,c,d). It allows for studying the physicochemical properties of the membranes.

Development of measurement technique

The requirements concerning the electrode impedance increase with increasing applied frequency or capacitance, when the capacitance is measured by any method. The capacitance current flowing across the membrane attains the values capable to perturb the electrode function.

In the here described system, the current flowing through the electrodes is of constant absolute value but its direction varies. The capacitance variation does not modify the absolute current value. The measurement

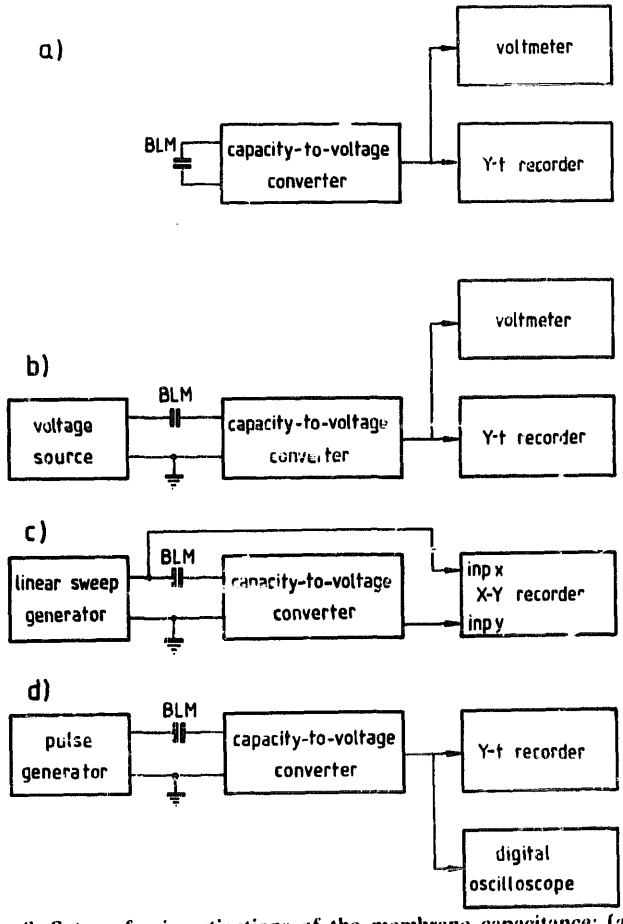


Fig. 8. Setups for investigations of the membrane capacitance: (a) measurement and/or recording of the membrane capacitance, (b) measurement and/or recording of the membrane capacitance with a membrane potential, (c) recording of the membrane capacitance as a membrane potential function, (d) recording of the capacitance of the membrane, which is excitated by voltage pulses.

error due to the electrode resistance changes insignificantly during the measurements.

At present, work is continued on elaboration of a new four-electrode system of capacitance measurement. Two control electrodes for measuring the potential difference and two electrodes for stimulating the current flow will make it possible to use high-resistance microelectrodes.

Device applications

Reagents, materials and methods

The lipid bilayers were formed using chromatography grade egg yolk lecithin, bovine brain phosphatydylserine (BDH), and the human erythrocyte membrane extract. The extract was prepared as follows: the blood conserved with the ACD liquid was centrifuged, the separated erythrocytes were washed three times with isotonic NaCl solution and centrifuged. The washed and and centrifuged erythrocytes were haemolyzed by placing them in distilled water. The erythrocyte ghosts obtained in this way were washed and centrifuged three times. The erythrocyte ghost lipids were extracted with a chloroform/methanol mixture (4:1, v/v). The solvents were removed from the extract using a cold air stream. The extract composition was studied by thin-layer chromatography.

The solvents used for forming solutions were distilled twice. The electrolyte was prepared using analytical grade KCl, KBr and KI (POCh) dissolved in twice distilled water.

The membranes were formed in a measuring vessel consisting of two chambers of 8 cm^3 volume each. The chambers were separated by a teflon or cellulose acetate septum with a hole of 1 mm diameter where the membrane was formed. Silver-silver chloride electrodes (a coiled wire 0.5 mm in diameter 8 cm long) were immersed in the electrolytes. The total impedance of the electrodes and the 0.1 M KCl solution in the measuring vessel was about 700 Ω. The electrodes were connected to the apparatus by a coaxial cable with capacitance of about 50 pF.

The forming solution drop (0.5–2 μl) was placed in the hole by means of a micropipette or of a 5 μl Hamilton microsyringe.

The capacitance measurements were carried out with two measuring systems. One of them consisted of the capacity-to-voltage converter described in the chapter Description of the instrument. The membrane capacitance was recorded with Czechoslovak BAK 5T or TZ4620 recorders. The polarization voltage source was an Elpan EG 20 type generator.

The other system consisted of a PC/AT computer, an interface with a capacity-to-period converter (Fig. 2), and a digital-analog converter for membrane polarization. The system with the computer and suitable software made it possible to carry out the measurements more rapidly, to store the experimental data on the disk and to draw the results with a plotter.

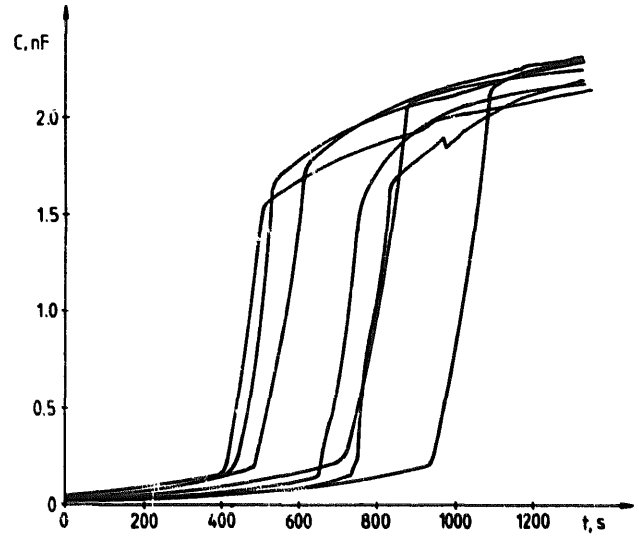


Fig. 9. Capacitance during formation of the membranes from lecithin 16 mg/cm^3 in mixture decane/butanol/chloroform (4:1:2, v/v), electrolyte 0.1 M KCl, septum: cellulose acetate.

Membrane capacitance during bilayer formation

The capacitances of the membranes being formed from lecithin solution in decane, chloroform and butanol are presented in Fig. 9. The septum (0.15 mm thick) was made of cellulose acetate. The membrane were formed the same day from the same solution. The shapes of all the curves are the same but time shifts can be observed. Bowed on capacitance curves of the membrane formation, the membrane formation process can be divided into three stages. A slow, slight capacitance increase occurs at the first stage. The second stage: a rapid capacitance increase begins. At the third stage the capacitance increases again but slowly and after a time period the capacitance attains a constant value. This capacitance does not charge during all the membrane lifetime by more than 5%. The capacities of the formed membranes presented in Fig. 9 are similar. The time period where the rapid capacitance increase begins is the most unreproducible membrane formation parameter.

The capacitances of membranes formed from lecithin dissolved in n-heptane of concentrations 6, 12.5 and 25 mg/ml are presented in Fig. 10. During 50–200 s since the drop of forming solution has been placed in the hole the membrane capacitance insignificantly increases and then a rapid capacitance increase begins. However, the capacitance increase is not continuous at this stage, the increases are followed by abrupt capacitance drops. After a time period (not shown in the figures) the capacity stabilizes. The capacitance stabilization is attained after a shorter time for more concentrated lecithin solution. The membranes

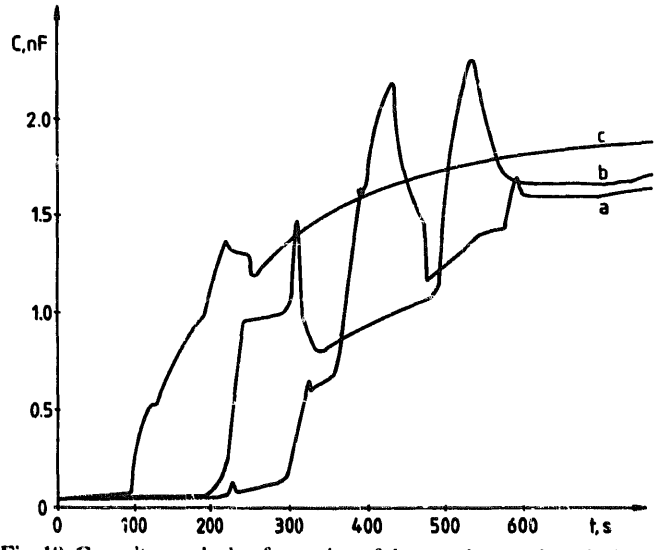


Fig. 10. Capacitance during formation of the membranes from lecithin dissolved in heptane, electrolyte 0.1 M KCl, septum: teflon. Lipid concentration: (a) 6 mg/cm^3, (b) 12.5 mg/cm^3, (c) 25 mg/cm^3.

formed from less concentrated solutions break down after a shorter time period.

Several capacitance curves of membranes during their formation where different solvents and different lecithin concentrations were used to prepare the membrane-forming solution are presented in Fig. 11. Considerable differences are present between the individual curves in the time needed to start capacitance increase, capacitance increase rate and capacitance stability of the formed bilayer.

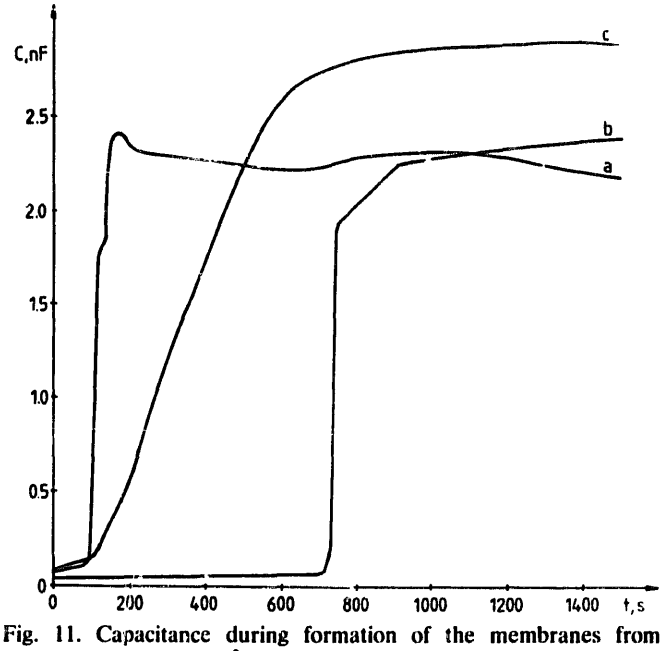


Fig. 11. Capacitance during formation of the membranes from lecithin: (a) 25 mg/cm^3 in heptane/isopropanol (1:1, v/v), electrolyte: 0.1 M KCl, septum: teflon; (b) 20 mg/cm^3 in hexane/heptane/isobutanol (5:2:5, v/v), electrolyte: 0.1 M KCl, septum: teflon; (c) 16 mg/cm^3 in octane/chloroform (5:1, v/v), electrolyte: 0.1 M KCl, septum: cellulose acetate.

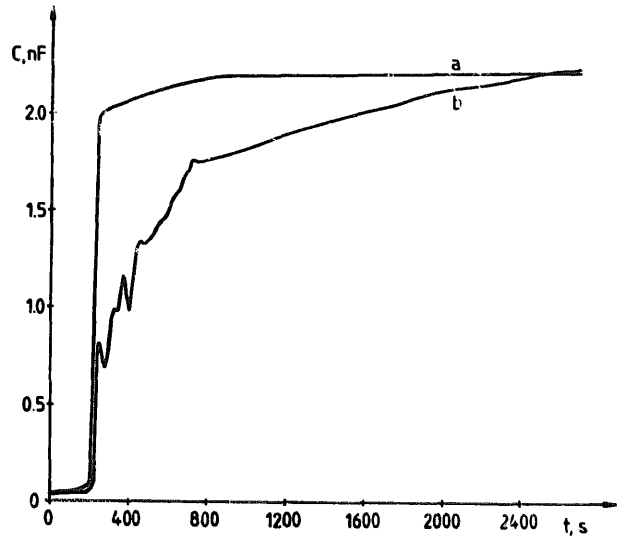


Fig. 12. Capacitance during formation of the membranes from (a) lecithin 8 mg/cm^3 and phosphatidylserine 12 mg/cm^3 in mixture decane/butanol (4:1, v/v), electrolyte 0.1 M KCl, septum: teflon; (b) human erythrocyte membrane lipids 20 mg/cm^3 in octane/butanol (4:1, v/v), electrolyte 0.1 M KCl, septum: teflon.

The capacitance curve for membrane formation from a lecithin-phosphatidylserine mixture is presented in Fig. 12a. The shape of the curve does not differ clearly from that of the membrane formed by lecithin alone under the same conditions.

There are the differences if the lipids extracted from human erythrocyte membranes are used (Fig. 12b). The stage of rapid capacitance increase is short and the stage of membrane capacitance stabilization lasts long.

Several characteristic stages can be distinguished lipid bilayer formation (Fig. 13):

(A) The membrane is initially thick when the drop of forming solution is placed in the hole. For this reason, its capacitance is low. Then the solvent and the excess lipid pass into the aqueous solution. The membrane thickness decreases with time, its capacitance increases slowly but steadily.

(B) At a moment the hydrocarbon chains of two opposite surface lipid layers touch one another. The hydrophobic interactions of these layers make the bilayer area increase. The lipid layers attracting themselves press the solvent and the excess lipid out to the hole edge. The membrane cross-section assumes a 'bone-like' shape.

(C) The rapid capacitance increase is stopped when the forces making the membrane expand are equilibrated by the membrane tension. The rate of further increase in the membrane capacitance is then determined by the rate of transfer of the solvent and of the excess lipids to the aqueous phase. The increase in the bilayer area is then slow and becomes still slower with elapsing time.

(D) When the solvent and excess lipid elimination from the membrane stops then the membrane capacitance does not increase any more. The membrane can be considered to be formed.

It can be estimated on the ground of the recorded capacitance curves of bilayer lipid membrane formation that the process is influenced to the highest degree by the solvent used for preparing the forming solution.

The membranes can be formed from the solution of lipids in an n-hydrocarbon. The stage B is highly unstable in the case of light hydrocarbons. The forces acting between the solvent and the lipid hydrocarbon chains are similar to those between the hydrocarbon chains of a bilayer. Therefore, there is no marked energy difference between two contacting monolayers and the monolayers separated by a non-polar solvent layer. If a polar solvent (an alcohol, chloroform) is present in the forming solution then the contact of two monolayers by their hydrocarbon chains is energetically favourable. It is expressed by a rapid and constant capacitance increase at stage B.

The solvents used most readily at present are the mixtures of decane with chloroform or butanol.

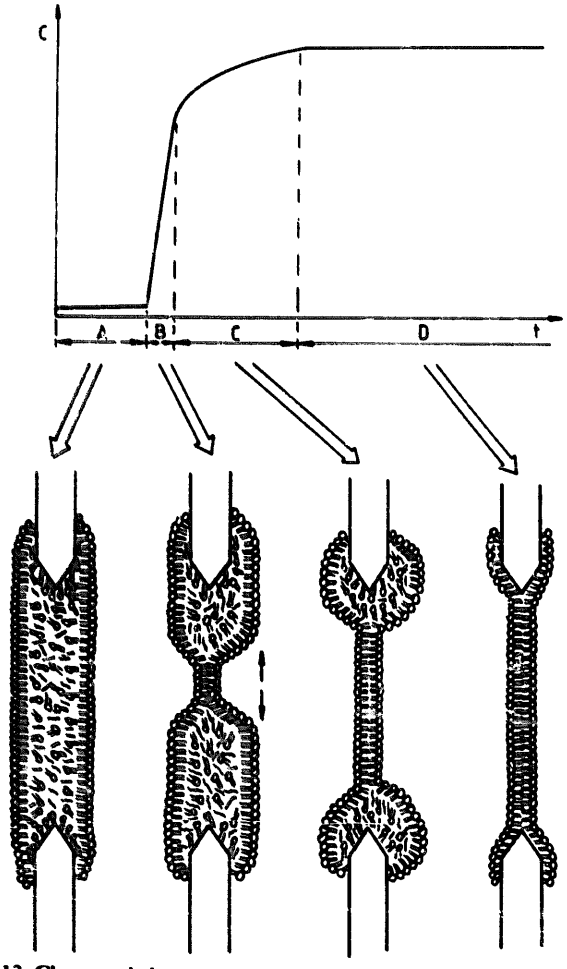


Fig. 13. Characteristics steps of bilayer lipid membrane formation.

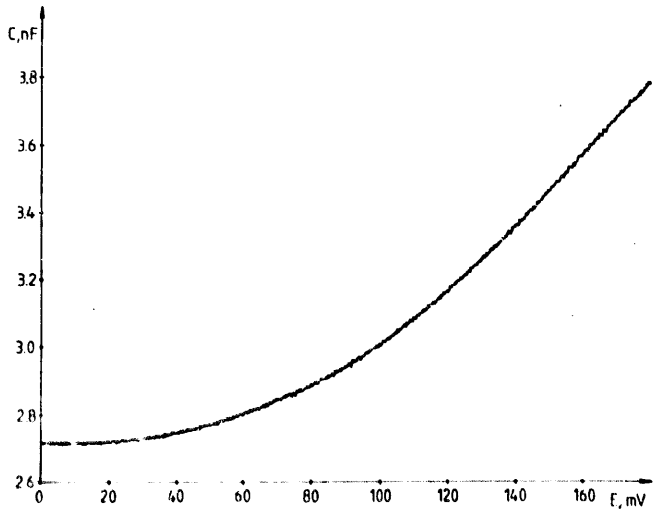


Fig. 14. Influence of the linear sweep voltage on the membrane capacitance. Forming solution: lecithin 10 mg/cm^3 in decane/chloroform (4 : 1, v/v), electrolyte: 0.1 M KCl, septum: teflon, voltage sweep speed: 10 mV/s.

An essential factor influencing the formation rate and the durability of formed membranes is a good wetting of the hole edge the membrane is spread in from the forming solution. Addition of chloroform to the forming solution has provided advantageous when a cellulose acetate foil was used as partition. It causes a good wetting by the solution and, mixed with a hydrocarbon, it does not dissolve the partition.

The duration of the stage A where the membrane thickness decreases depends on the solvent volatility. The bilayer is formed rapidly when the forming solution contains light hydrocarbons (hexane, heptane, octane) but a droplet of rapidly evaporating liquid can appear in the hole.

Polarization potential influence on the membrane capacitance

The effect of linearily changing potential on the membrane capacitance is presented in Fig. 14. At potentials over 120 mV, the capacitance increases linearily with the potential. The capacitance increase in undetectable at low potentials. The capacitance of cyclic polarized membrane is presented in Fig. 15.

The effect of a 100 mV voltage pulse of 4 s duration on the lecithin membrane capacities is presented in Fig. 16. The membranes were formed in the presence of electrolytes containing KCl, KBr, or KI. Silver wire electrodes electrolytically covered with AgCl, AgBr, and AgI, respectively, were placed in the solutions. The greatest capacitance change occured for the electrolyte containing the I^- ions. The capacitance variation rate resulting from the potential change is relatively low.

The effect of a rectangular voltage pulses sequence on the membrane capacitance is presented in Fig. 17.

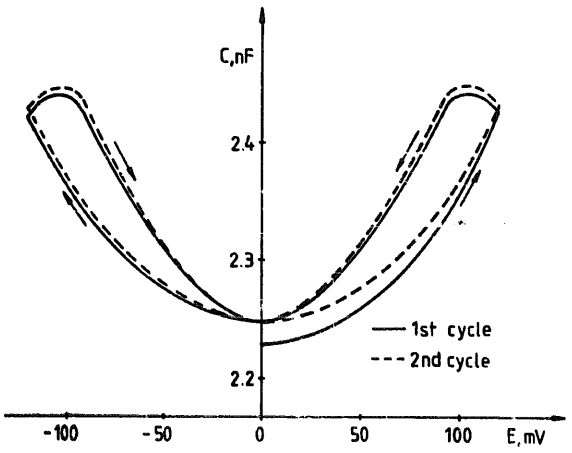


Fig. 15. Influence of the cyclic linear sweep voltage on the membrane capacitance. Forming solution: lecithin 25 mg/cm^3 in heptane/butanol (3:1, v/v), electrolyte: 0.1 M KCl, septum: teflon, voltage sweep speed: 10 mV/s.

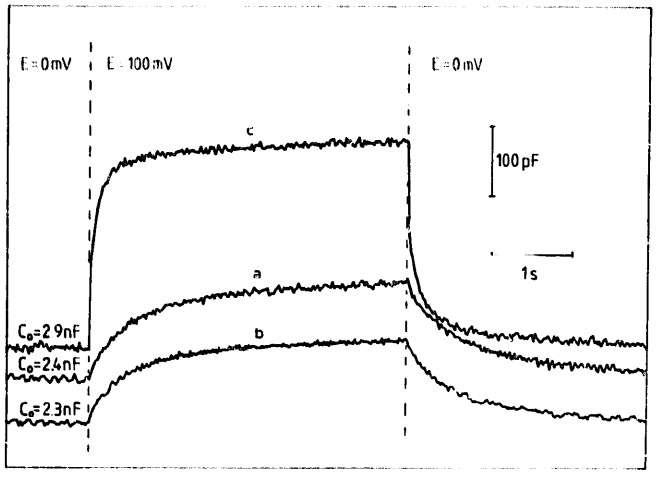


Fig. 16. Influence of the voltage pulses on the membrane capacitance. Forming solution: lecithin 10 mg/cm^3 in octane/butanol (3:2, v/v), septum: teflon, electrolytes: (a) 0.5 M KCl, (b) 0.5 M KBr, (c) 0.5 M KI.

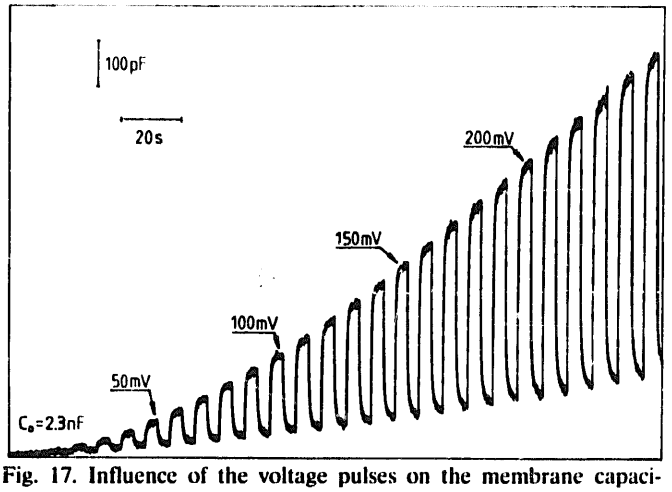


Fig. 17. Influence of the voltage pulses on the membrane capacitance. The voltage of the succeding pulses increased 10 mV. Pulses width: 4 s, distances between pulses: 4 s, forming solution: lecithin 10 mg/cm^3 in octane/butanol (3:2, v/v), electrolyte: 0.1 M KCl, septum: teflon.

The pulses were 4 s wide and intervals between them amounted to 4 s, too. The voltage pulses increased by 10 mV at each step. The information obtained from Fig. 17 is similar to that from Figs. 14 and 16.

The effect of polarization potential on the bilayer lipid membrane capacitance was earlier studied by Benz et al. [14]. The phenomenon was explained in terms of membrane thickness and area variations. The investigations of capacity change kinetics and the study of the effect over a broader potential range do not preclude the essential role of the electric permittivity variations of the hydrophobic membrane part. Further studies are required to explain the phenomenon in more detail.

Conclusions

The instrument developed by us has proven to be handy and to be useful in the studies of membrane phenomena. Its first application was the recording of membrane capacitances in the process of their formation by the classical method. It allowed for estimating whether the membrane was already formed and ready for further studies. The instrument accompanied by other devices (Fig. 8) yields sets making it possible to study various membrane phenomena. The instrument performance is good having in view its cost.

The coupling of the capacitance transducer with a computer yields a measuring system which makes it possible to carry out quickly and simply the measurements which are hard to do with a classical apparatus.

The forming solution composition is an essential factor affecting the membrane formation process. The possibility to record the capacitance of forming membrane permits to choose the forming solution composition so as to obtain rapidly forming and reproducible bilayer lipid membranes. The knowledge of the membrane formation process facilitates the interpretation of other experimental results obtained with the membrane.

Little attention has been paid hitherto to the studies of bilayer lipid membranes. The hydrophobic part electric permittivity which determines the membrane capacitance is low, 2.2–2.6 [1]. Insignificant structural changes of this area and the presence of alien molecules or ions can cause serious changes in the membrane capacitance. The capacitance measurements can contribute to large degree to the experimental studies based on classical voltamperometric techniques.

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