

## Structural Changes of Excitable Membrane Formed on the Surface of Protoplasmic Drops Isolated from *Nitella*

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*Summary.* The protoplasmic drop isolated from *Nitella* became electrically excitable in an appropriate salt solution. Variations of salt composition and temperature in the external media induced discrete changes in the interfacial properties such as refractive index or surface tension, as well as in the electrical properties of the protoplasmic drop. Variation of salt composition was performed by changing the concentration of the respective component at a time from the original test solution (0.5 mM NaCl, 0.5 mM KNO<sub>3</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, and 2 mM Mg(NO<sub>3</sub>)<sub>2</sub>) with other components being fixed. The following results were obtained: (1) An increase of concentration of univalent cation (Na<sup>+</sup> or K<sup>+</sup>) induced a sharp rise of the refractive index from 1.42 to 1.58 at about 10 mM, which was accompanied by a discrete depolarization of the membrane potential of the drop. Fluctuation of reflected light was observed at the critical concentration. (2) The refractive index remained at low values of about 1.42 in Ca<sup>2+</sup> concentration ranging between about 0.1 and 10 mM. Beyond this concentration range of Ca<sup>2+</sup>, i.e., both lower and higher, the refractive index increased discontinuously to the value of about 1.58. (3) An addition of multi-valued cations in the test solution led to an abrupt depolarization of the membrane potential at a definite concentration for each ion species. The critical salt concentration depended strongly on the valence of cations added, and gave the following series: Th<sup>4+</sup> < La<sup>3+</sup> < Ba<sup>2+</sup>, Sr<sup>2+</sup> ≲ Na<sup>+</sup>. (4) Lowering of the surrounding temperature induced an abrupt depolarization of the membrane potential when the Ca<sup>2+</sup> concentration in the external solution was close to the lower critical concentration, i.e. 0.1 mM. (5) A rise in temperature led to an increase of fluctuations of the membrane potential with frequent outbreaks of small spike potentials, which were followed by an abrupt depolarization. At the same time the tension at the surface decreased discontinuously from 10<sup>-1</sup> dynes/cm to the order of 10<sup>-4</sup> dynes/cm. All these changes were reversible.

These results imply that the surface membrane of the protoplasmic drop is liable to change its structure in response to the variation of temperature, salt composition in the external media and/or electrical stimulus. Drastic variations of membrane properties such as surface tension, membrane potential and membrane resistance are discussed in connection with the structural change determined by the refractive index measurements.

In a preceding series of papers [4, 7, 10, 12], we reported some physico-chemical properties of the excitable membrane formed on the surface of protoplasmic drops isolated from the internodal cell of *Nitella*. The fact that the surface membrane is contiguous to external solution is a great merit of

the system under study. This permits the determination of surface tension and refractive index together with the electrical properties of the membrane. It was demonstrated that the variation of external salt composition induced sharp changes of the surface tension, membrane potential and membrane resistance, which were accompanied with occurrence or disappearance of excitability of the drop membrane. These results imply that there exist at least two distinct states in the membrane conformation.

Transition between two steady states of the surface membrane of the drop may be induced by various kinds of physical and chemical factors in the surrounding media, e.g., ionic strength in the external solution, temperature, electric current, etc.

In this paper we report the transition of the membrane structure caused by various kinds of physical factors in the media. The structural changes are detected by measurements of the refractive index, the surface tension as well as electrical properties of the drop membrane. From the results obtained, possible physical structures of the two states of the surface membrane are discussed.

### Experimental

Experimental procedures were essentially the same as those employed previously [7, 12]. An internodal cell of *Nitella flexilis* was amputated in a solution containing 70 mM KNO<sub>3</sub>, 50 mM NaCl and 5 mM Ca(NO<sub>3</sub>)<sub>2</sub> [8]. The effused protoplasmic drop was allowed to stand in a test solution (0.5 mM NaCl, 0.5 mM KNO<sub>3</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub> and 2 mM Mg(NO<sub>3</sub>)<sub>2</sub>; the solution was buffered at about 6.5 pH by Tris acetate and was made isotonic by adding mannitol). The protoplasmic drop which reached the steady state in the test solution was found to be electrically excitable. Variation of composition in the external solution from the test solution described above was performed after the drop attained the steady state.

All the experiments were performed at room temperature, 20 ± 2 °C, except in the experiment on temperature effect. The change in temperature was performed by perfusing the cold or hot solution in the external solution, or by using a cooling jacket attached to the measuring cell. Temperature was measured by a pair of thermocouples (Cu-constantan).

Materials used were ThCl<sub>4</sub>, LaCl<sub>3</sub>, BaCl<sub>2</sub>, SrCl<sub>2</sub>, Mg(NO<sub>3</sub>)<sub>2</sub>, Ca(NO<sub>3</sub>)<sub>2</sub>, KNO<sub>3</sub>, NaCl and Tris acetate of analytical grade. Mannitol was recrystallized from ethanol.

#### *Determination of Refractive Index at the Surface of Protoplasmic Drops*

Apparatus and arrangement used for the determination of the Brewster angle  $\alpha$  were the same as those reported in a previous paper [7], except for the light source. The light source was a He-Ne laser, having the output power of 1.5 mW (CW Radiation, Inc., Model S-203).

Refractive index  $n$  at the surface of protoplasmic drop was calculated by using the following equation:

$$n = n_s \tan \alpha$$

where  $n_s$  is the refractive index of the external medium.

*Determination of Tension at the Surface*

Tension at the surface  $\gamma$  of protoplasmic drop was determined either by sessile drop [1, 9] or by a compression method. Experimental details were described previously [12].

**Results and Discussion***Effect of Univalent Cations*

Fig. 1 shows the concentration dependence of the refractive index  $n$  at the surface of protoplasmic drops, where compositions other than NaCl or  $\text{KNO}_3$  were fixed at 1 mM  $\text{Ca}(\text{NO}_3)_2$  and 2 mM  $\text{Mg}(\text{NO}_3)_2$ . Increase of concentration of univalent cations induces a sharp rise of  $n$  from 1.42 to 1.58 at about 10 mM  $\text{Na}^+$  or  $\text{K}^+$ . These results indicate that a structural change takes place at the surface of protoplasmic drops of *Nitella*. The following discussion will make clear the nature of the structural change of the surface membrane.

First, we notice that the refractive index gives unexpectedly high values of about 1.6 in a high concentration media. On this point, it has been reported that determination of  $n$  in black lipid membrane from the Brewster

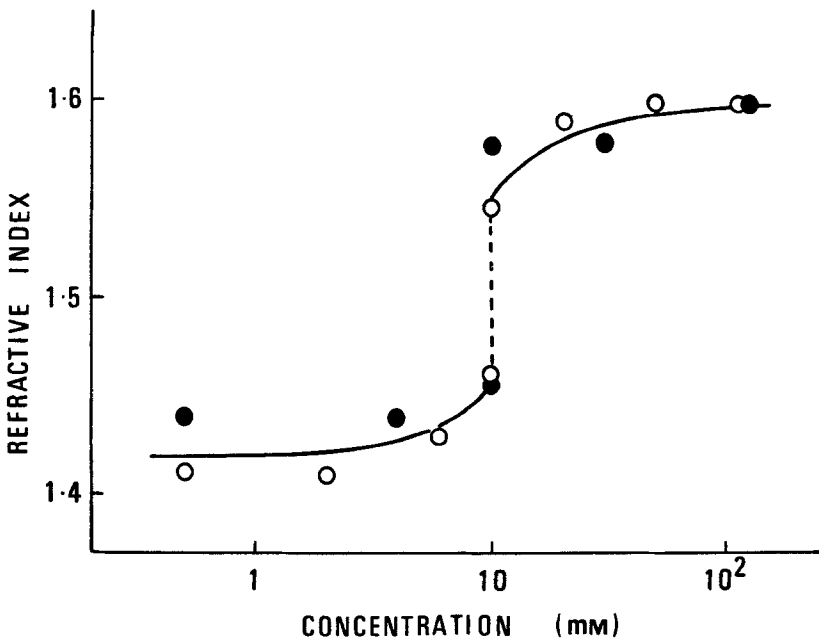


Fig. 1. Dependence of the refractive index at the surface of protoplasmic drops on  $\text{K}^+$  and  $\text{Na}^+$  concentrations. Open circles:  $\text{KNO}_3$ ; closed circles: NaCl. Concentrations of other components were fixed at 1 mM  $\text{Ca}(\text{NO}_3)_2$  and 2 mM  $\text{Mg}(\text{NO}_3)_2$ .

angle measurements gives a value as high as 1.66 [3], which is much higher than that expected from the refractive index of component phospholipids in the bulk phase. This fact suggests that a high value of  $n$  may be a characteristic of a bilayer lipid membrane, although there are inherent difficulties in applying Brewster's angle measurements to ultrathin structures such as black lipid membranes. In addition, electron microscopic study [5] seems to support the view that surface membrane may be a bimolecular lipid membrane in high ionic concentration media.

However, drop membrane has low electric resistance (about  $0.1 \text{ k}\Omega \text{ cm}^2$ ) [4, 7, 10] and high cation permeability at high concentrations (N. Ishida, T. Kashiwakura, I. Inoue & Y. Kobatake, *unpublished data*). These findings indicate that drop membrane behaves like a charged membrane with negative fixed charges [11], and deny the view that drop membrane would be a compact and uniform bilayer lipid membrane as in an artificial one. A structure at the surface of the drop which is inferred from the results described above is as follows: Micelles of phospholipids are loosely bound with each other at the surface of protoplasmic drop. For the sake of convenience, in further discussion we will refer to this kind of lipid-rich surface structure as "lipid mosaic".

Second, we mentioned the low values of  $n$  at low concentrations. In a previous paper [7], we showed that the decrease of  $n$  was attributed to the penetration of proteins into surface membrane from inner protoplasm by studying the actions of proteases on the electrical properties of the surface membrane. Therefore, a low value of  $n$  is a reflection of the formation of lipid-protein complex in the surface membrane. Thus, we may interpret the sharp variation of  $n$  at about  $10 \text{ mM Na}^+$  or  $\text{K}^+$  as a transition in the membrane structure between "lipid mosaic" and lipid-protein complex.

It was noticed that reflected light flickered in a period of a few seconds at the critical concentration. This observation may also support the view that there occurs a transition of the membrane structure, because fluctuation in macroscopic level of a physical property is well known in critical phenomena. A macroscopic fluctuation in membrane potential was also recorded near the abrupt depolarization of a drop membrane [4, 11].

Structural change of the surface membrane may give rise to a discrete alteration of physical properties of the membrane. Tension at the surface of protoplasmic drop decreased sharply from  $0.1 \text{ dyne/cm}$  to  $0.003 \text{ dyne/cm}$  at about  $10 \text{ mM}$  by increasing the concentration of  $\text{Na}^+$  or  $\text{K}^+$  [12]. Increase of  $\text{Na}^+$  concentration induced an abrupt depolarization of membrane potential and a discrete fall of membrane resistance around  $10 \text{ mM}$ . The excitability appeared only at low concentration media; i.e. below  $10 \text{ mM Na}^+$

or  $K^+$  [4, 7, 10]. All these results indicate that concentration of univalent cations is one of the factors which influences the transition in the membrane structure between "lipid mosaic" and lipid-protein complex. But it may be important to note that the decrease of external salt concentration brings simultaneously large differences in concentration and in the potential across the membrane. These nonequilibrium factors may play some roles in the formation of the excitable membrane.

### *Effect of $Ca^{2+}$ Concentration*

Open circles in Fig. 2 show the dependence of  $n$  on the  $Ca^{2+}$  concentration, where other components were fixed at 0.5 mM NaCl, 0.5 mM  $KNO_3$  and 2 mM  $Mg(NO_3)_2$ . In marked contrast to the case of univalent cations, discrete changes of  $n$  appeared at two points; i.e., at about 0.1 and 10 mM  $Ca(NO_3)_2$ . In the concentration range between 0.1 and 10 mM  $Ca^{2+}$ , the refractive index gives the low value of about 1.42. Beyond this concentration of  $Ca^{2+}$  (lower and higher), the refractive index rises discontinuously to about 1.56. Discrete increases of  $n$  at two critical concentrations may be attributed to the destruction of lipid-protein complex in the membrane as in the case of univalent cations.

Closed circles in Fig. 2 indicate the values of  $n$  where the concentration of NaCl is fixed at 30 mM. High concentration of univalent cation prevents the decrease of  $n$  by varying  $Ca^{2+}$  concentration and gives a high  $n$  value of

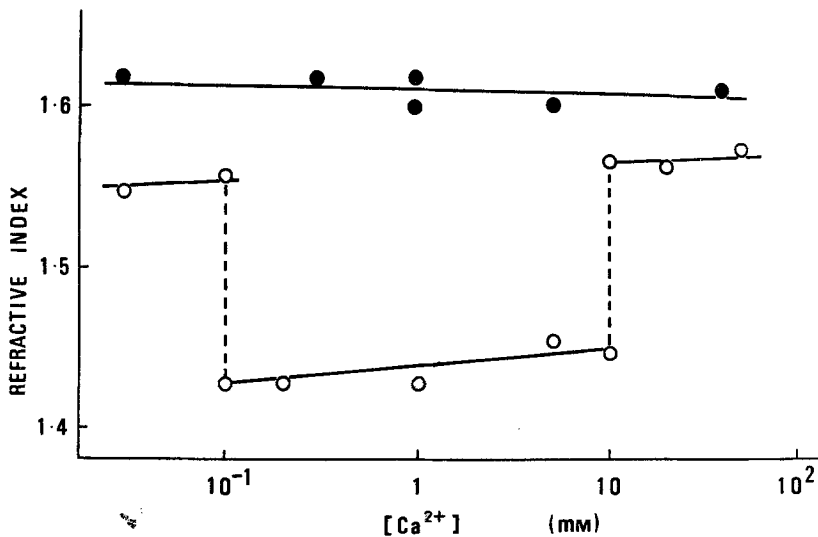


Fig. 2. Dependence of the refractive index at the surface of protoplasmic drops on  $Ca^{2+}$  concentration. Open circles: 0.5 mM NaCl, 0.5 mM  $KNO_3$ , and 2 mM  $Mg(NO_3)_2$ . Closed circles: 30 mM NaCl, 0.5 mM  $KNO_3$  and 2 mM  $Mg(NO_3)_2$

about 1.6, which is characteristic to that of "lipid mosaic" structure at the surface. We have already reported [3] that high concentration of univalent cations gave a linear relationship between  $\log \gamma$  and  $\log (\text{Ca}^{2+})$ , and that the curves follow the same straight line both in higher and lower concentrations of  $\text{Ca}^{2+}$  although plots of  $\log \gamma - \log (\text{Ca}^{2+})$  remain almost constant in the concentration ranging between 0.1 and 10 mM of  $\text{Ca}^{2+}$ . Here  $\gamma$  indicates tension at the surface of protoplasmic drop. These facts support the idea that structures of surface membrane appearing in low and high concentration of  $\text{Ca}^{2+}$  are identical with each other; i.e., "lipid mosaic".

The transition observed on the low concentration of  $\text{Ca}^{2+}$  may be interpreted as the structural change caused by a release of membrane-bound  $\text{Ca}^{2+}$ . However, this mechanism of transition is not applied to the transition occurring in high  $\text{Ca}^{2+}$  concentration. Dual roles specific to  $\text{Ca}^{2+}$  will be made clear by the following experiments.

#### *Effect of Polyvalent Cations*

Fig. 3 shows the dependence of the membrane potential on the concentration of various salts of polyvalent cations; i.e.,  $\text{ThCl}_4$ ,  $\text{LaCl}_3$ ,  $\text{SrCl}_2$ ,

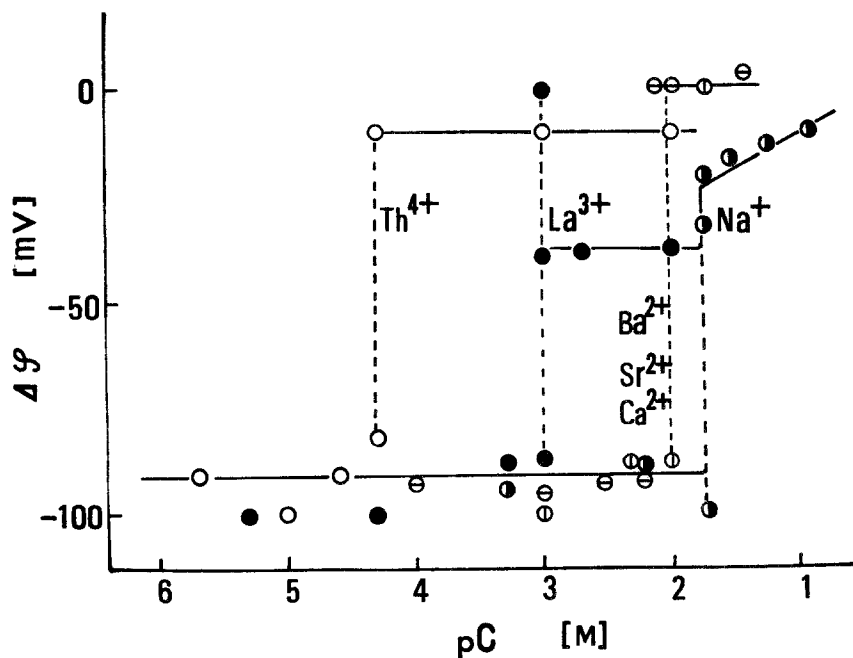


Fig. 3. Concentration dependence of the membrane potential for various salts of different valencies.  $\circ$ ,  $\text{ThCl}_4$ ;  $\bullet$ ,  $\text{LaCl}_3$ ;  $\ominus$ ,  $\text{BaCl}_2$ ,  $\text{SrCl}_2$  and  $\text{Ca}(\text{NO}_3)_2$ ;  $\odot$ ,  $\text{NaCl}$ . Each salt is added successively in the test solution (see Text)

BaCl<sub>2</sub>. Abrupt depolarization occurs at a certain concentration for respective cations, when the concentration is increased gradually in the external solution. Note that the critical concentration for the abrupt depolarization depends strongly on the valence of cations involved:  $5 \times 10^{-5}$  M for Th<sup>4+</sup>,  $1 \times 10^{-3}$  M for La<sup>3+</sup>, and  $1 \times 10^{-2}$  M for Ba<sup>2+</sup>, Sr<sup>2+</sup> and Ca<sup>2+</sup>. This fact indicates that the membrane structure is greatly affected by the ionic strength factor. It is important to note that the membrane potential is not depolarized when these polyvalent cations are decreased successively as was observed in the case of Ca<sup>2+</sup>. Therefore we may conclude that Ca<sup>2+</sup> in the external solution takes two different roles in the transition of the membrane structure; i.e., by functioning as a specific binding reagent and as a non-specific ionic strength factor. It is not necessary to consider that these dual functions of Ca<sup>2+</sup> act at the same specific site on the membrane. The changes in the membrane properties caused by ionic composition in the media were reversible for most cases unless the drop was allowed to stand for a long time in the depolarized state. For example, when the drop was placed in a solution of 10 mM ThCl<sub>4</sub> for 10 min, the protoplasmic movement in the drop was suppressed and led to an irreversible change of the drop. The similar, but strictly speaking, slightly different toxic effects are observed for various inorganic poisons such as UO<sub>2</sub><sup>2+</sup>, Fe<sup>3+</sup>, Hg<sup>2+</sup>, Cu<sup>2+</sup>, Cd<sup>2+</sup>, Ag<sup>+</sup>, etc. on the membrane properties of the drop as will be reported elsewhere.

#### *Transition Induced by Temperature Change*

Transition of the membrane structure is also induced by the temperature variation in the medium. Decrease in temperature caused an abrupt depolarization when the composition of the external solution was kept constant near the critical salt concentration. Critical temperature in a fixed salt composition differed from one drop to another and depended on the external salt composition. Fig. 4 shows an example of the time-course of the membrane potential when the temperature in the external solution was lowered and kept at 4 °C. Here the concentration of Ca<sup>2+</sup> was 0.2 mM and other salts were the same as those in the test solution. Membrane potential was depolarized suddenly by more than 50 mV in the low temperature.

It is well known that the nerve excitation is accompanied by an evolution of heat [2], and that the lowering of temperature induces an abrupt depolarization of the membrane potential for an internally perfused squid axon [6]. The internodal cell of *Nitella flexilis* is also depolarized abruptly by lowering the temperature. These reports as well as the result shown in Fig. 4 seem to imply that the excitation process in protoplasmic drop of *Nitella* is an exothermic reaction. This implication must, however, be postponed until a

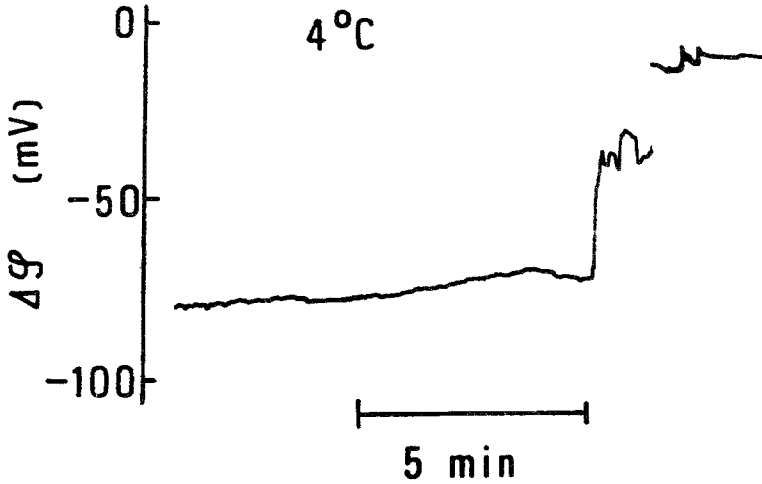


Fig. 4. Time-course of the membrane potential at low temperature. The external solution was cooled to 4 °C. Salt concentrations were the same as those of the test solution except  $\text{Ca}^{2+}$  which was 0.2 mM

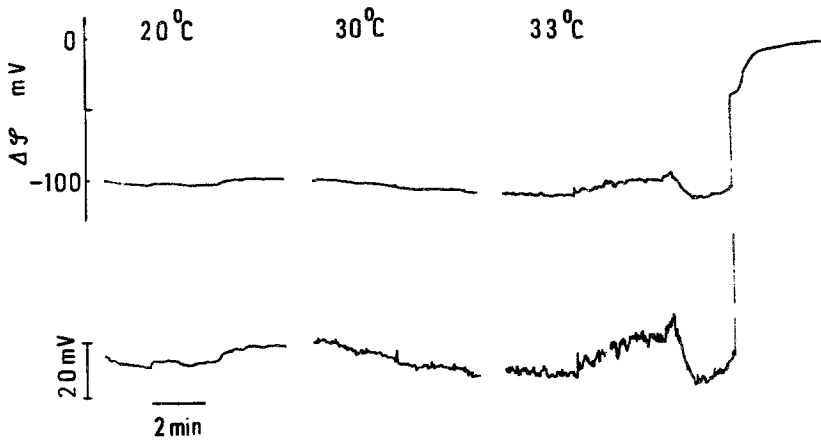


Fig. 5. Transition and fluctuation of the membrane potential in high temperature. Temperature was raised gradually as indicated in the figure. The composition of the solution was the same as that of the test solution

direct evidence is obtained from the drop, because the rise in temperature also induces an abrupt transition in membrane potential. Fig. 5 shows the time-course of the membrane potential as the temperature was raised successively. Lower traces of the figure show the magnified oscilloscope traces of the membrane potential. The fluctuation of the membrane potential increased gradually as the temperature approached the critical point. Immediately before the transition, the outbreak of the spike potentials of a



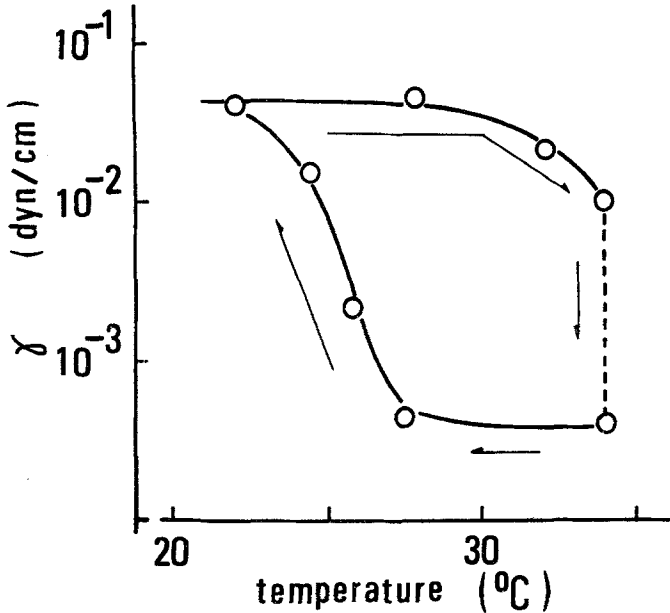


Fig. 6. Hysteresis in the tension at the surface with a cyclic change in the temperature. A period of approximately 15 min was required to complete one cycle of temperature change

few millivolts in amplitude became frequent. A spontaneous firing of action potentials became frequent in an internodal cell of *Nitella* as the temperature increased.

It is interesting to note that the abrupt depolarization of the membrane potential is accompanied by a sudden decrease of tension at the surface of the drop. Fig. 6 shows the temperature dependence of tension at the surface of a drop where temperature was changed successively and repeatedly between 20 and 35 °C. One cycle required about 15 min. Tension at the surface decreased discontinuously from  $5 \times 10^{-2}$  to  $3 \times 10^{-4}$  dynes/cm at about 34 °C. Surface tension recovered when the temperature in the external solution was lowered to room temperature.

All the results described above indicate that the excitable surface membrane of protoplasmic drop is liable to change its structure more or less discontinuously and abruptly when the environmental conditions are changed; e.g., composition and temperature in the external solution.

The next problem is to determine what kind of molecular interaction participates in maintaining the structure of the excitable membrane. A study along this line is now in progress.

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## References

1. Dorsey, N. E. 1928. A new equation for the determination of surface tension from the form of a sessile drop or bubble. *J. Wash. Acad. Sci.* **18**:505
2. Howarth, J. V., Keynes, R. D., Ritchie, J. M. 1968. The origin of the initial heat associated with a single impulse in mammalian non-myelinated nerve fibres. *J. Physiol.* **194**:745
3. Huang, C., Thompson, T. E. 1965. Properties of lipid bilayer membranes separating two aqueous phases: Determination of membrane thickness. *J. Mol. Biol.* **13**:183
4. Inoue, I., Ishida, N., Kobatake, Y. 1973. Studies of excitable membrane formed on the surface of protoplasmic drops isolated from *Nitella*. IV. Excitability of the drop membrane in various compositions of the external salt solution. *Biochim. Biophys. Acta* **330**:27
5. Inoue, I., Ishima, Y., Horie, H., Takenaka, Y. 1971. Properties of excitable membrane produced on the surface of protoplasmic drop in *Nitella*. *Proc. Japan Acad.* **47**:549
6. Inoue, I., Kobatake, Y., Tasaki, I. 1973. Excitability, instability and phase transitions in squid axon membrane under internal perfusion with dilute salt solutions. *Biochim. Biophys. Acta* **307**:471
7. Inoue, I., Ueda, T., Kobatake, Y. 1973. Studies of excitable membrane formed on the surface of protoplasmic drops isolated from *Nitella*: I. Conformation of surface membrane determined from the refractive index and from enzyme actions. *Biochim. Biophys. Acta* **298**:653
8. Kamiya, N., Kurada, K. 1957. Cell operation in *Nitella*. I. Cell amputation and effusion of the endoplasm. *Proc. Japan Acad.* **33**:149
9. Kamiya, N., Kurada, K. 1958. Cell operation in *Nitella*. IV. Tension at the surface of the effused endoplasmic drops. *Proc. Japan Acad.* **34**:435
10. Miyake, M., Inoue, I., Kobatake, Y. 1973. Studies of excitable membrane formed on the surface of protoplasmic drops isolated from *Nitella*.<sup>1</sup>III. Impedance of the surface membrane. *Biochim. Biophys. Acta* **323**:369
11. Tasaki, I. 1968. Nerve Excitation. Charles C. Thomas, Springfield, Ill.; also, Kobatake, Y., Tasaki, I., Watanabe, A. 1971. Phase transition in membrane with reference to nerve excitation. *Advanc. Biophys.* **2**:1
12. Ueda, T., Inoue, I., Kobatake, Y. 1973. Studies of excitable membrane formed on the surface of protoplasmic drops isolated from *Nitella*. II. Tension at the surface of protoplasmic drops. *Biochim. Biophys. Acta* **318**:326