

## THE ELECTRICAL NATURES OF FROG SKIN FROM THE VIEWPOINT OF "TWO MEMBRANE THEORY"

YOSHIFUSA ITO

*2nd Department of Physiology, Nagoya University School of Medicine  
(Director: Prof. Ryo Ito)*

It is a well known fact that when the isolated surviving frog skin is in contact with appropriate solutions on both sides, the skin maintains a considerable potential difference, viz. the frog skin potential (F.S.P.), between the outside and the inside for many hours. Its magnitude varies considerably with the constitutions of the solutions on both sides (Hashida 1922, Motokawa 1935, Linderholm 1952).

By applying an external E.M.F. the potential difference across the skin can be maintained equal to zero. Then an electric current, namely, short-circuit current (S.C.C.) is generated in stead of F.S.P. The current density varies with the constitutions of the solutions. Even when Ringer solution is placed on both sides of the frog skin, such F.S.P. or S.C.C. develops. Ussing (1949, 1951) proved that Na ions, and only Na ions, are actively transported from the outside to the inside. Consequently, it was confirmed that the generation of either F.S.P. or S.C.C. is due to the active transport of Na ions across the frog skin from the outside to the inside.

Linderholm (1952) has proposed "one membrane theory" on the generation of F.S.P. or S.C.C., considering the active transport of Na ions. In order to treat these phenomena theoretically, he assumed that the frog skin is a homogeneous uncharged membrane. Garby and Linderholm (1954) claimed that the lipid-sieve theory or the pore theory is compatible with the ionic permeability of the frog skin.

Studying the electrical potential gradient through the frog skin which was bathed in sulphate Ringer by means of microelectrodes, Hoshiko and Engbaek (1957) found two, rarely three, distinct potential jumps. They concluded from their observations that the frog skin has two diffusion barriers existing in the regions of the epidermis and the basement membrane.

Ussing (1958, 1960) studied the electric potential of the frog skin which was treated with  $\text{Cu}^{++}$ , or immersed in sulphate Ringer. He found that the outward-facing surface behaved like a sodium electrode and the inward-facing surface like a potassium electrode.

In order to explain these results, Ussing proposed "two membrane theory" as follows;

- 1) The frog skin has two membranes as diffusion barriers. The inward-

facing membrane is permeable to K ions and the outward-facing membrane to Na ions.

2) The active pump is located at the inner membrane which can be considered as a forced exchange of Na ions from the cells against K ions from the inner solution.

Ussing's hypothesis seems to be the most appropriate to account for the natures of the frog skin in the various conditions.

The author has studied the electrical natures of the frog skin from the standpoint of "two membrane theory."

#### METHODS

The technique and the apparatus were similar to those used by Ussing and Zerahn (1951), and by Linderholm (1952). The isolated abdominal skin from a frog (*Rana nigromacrata*) was mounted as a membrane between two plastic chambers each with a 8 ml capacity (Fig. 1). The skin was in contact with the solutions through holes (A, A'; each diameter 1 cm).

The potential difference was measured with saturated KCl-caromel electrodes (B, B') whose tips were of sleeve-type.

The electric current was supplied through Ag-AgCl electrodes (C, C'). The electric conductance was estimated by changing current density. Namely, the potential difference (V) corresponding to the current density (I) was measured by using a valve-volt-

meter. The electric conductance (per  $\text{cm}^2$ ) was calculated from the  $\partial I / \partial V$ . A slight difference in the electric potential caused by the resistance between the skin and the electrode was always corrected by adjusting current density or by calculation.

Ringer solution, isotonic NaCl solution (pH 7.9) containing  $\text{NaHCO}_3$  and  $\text{NaH}_2\text{PO}_4$  as buffer, and isotonic KCl solution (pH 7.9) containing  $\text{KHCO}_3$  and  $\text{KH}_2\text{PO}_4$  as buffer were used as the immersing solutions. These solutions were diluted with distilled water depending on the purpose of examination. The solution was continuously bubbled by moist air.

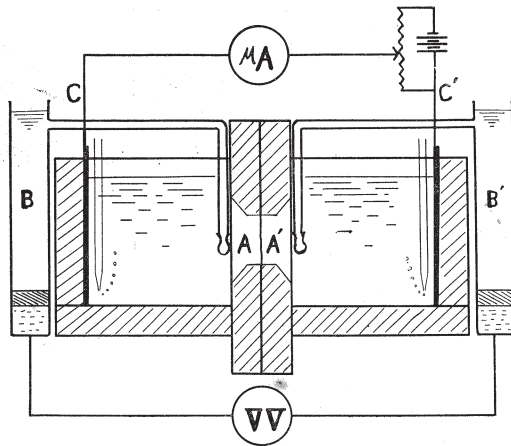


FIG. 1. Diagram of apparatus

- AA': Holes through which the frog skin was in contact with the solutions.
- BB': Saturated KCl-caromel electrodes.
- CC': Ag-AgCl electrodes used for applying the external E.M.F.
- VV: Valve-voltmeter.
- $\mu\text{A}$ : Microammeter.

## RESULTS

1. *Electric Resistance.* In Fig. 2, the electric resistance of the frog skin while Ringer solution was placed on both sides is compared with that while Ringer solution was placed on one side and the isotonic KCl solution on the other.

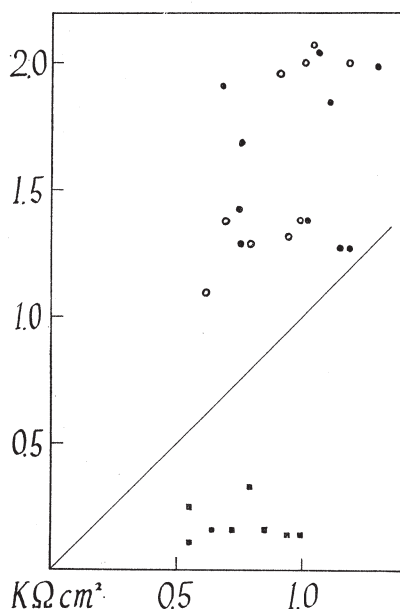


FIG. 2. The electric resistances of the frog skin under various ionic conditions. (see text).

Abscissa values (R-R values) of all symbols in Fig. 2 give the electric resistances of the frog skins against Ringer solution in both chambers, and ordinate value (R-K value) of solid square gives the electric resistance of the frog skin when Ringer solution in the inner chamber was replaced with the isotonic KCl solution. The measurements were done after the steady state had been reached. The average of R-R values of solid squares was calculated at  $0.75 \pm 0.16 \text{ K}\Omega\text{cm}^2$ . On the other hand, the average of R-K values of solid squares,  $0.18 \pm 0.02 \text{ K}\Omega\text{cm}^2$ , is distinctly smaller than the former. After the measurement of the latter, the KCl solution of the inside chamber was replaced with Ringer solution and the electric resistance was measured again. The electric resistance can be restored, if the inward-facing surface of the frog skin is in contact with KCl solution within 1 or 2 hours. Further contact

of the skin with KCl resulted in a incomplete recovery of the electric resistance. Such a irreversibility seemed to be due to deterioration of the inward-facing surface of the frog skin.

When Ringer solution of the outer chamber was replaced with the isotonic KCl solution, the electric resistance of the frog skin became higher. Ordinate values (K-R values) of solid circles and of open circles give the electric resistances in the situation that the inward-facing surface of the frog skin was in contact with Ringer solution and the outward-facing surface with the isotonic KCl solution. R-R and K-R values of solid circles were measured before and after the replacement of the outside Ringer solution with the isotonic KCl solution, while K-R and R-R values of open circles before and after the replacement of the outside KCl solution with Ringer solution. In both cases, the electric resistances were measured after the steady state had been reached.

No remarkable difference between the situations of solid circles and those of open circles was observed. This fact may be interpreted as that the isotonic

KCl solution is not harmful to the outside surface of the frog skin. For this reason, we adopted R-R and K-R values of open circles as well as those of solid circles for the interpretation of the data. The average of R-R values of solid circles and of open circles was calculated at  $0.96 \pm 0.20 \text{ K}\Omega\text{cm}^2$ . On the contrary, the average of K-R values was calculated at  $1.72 \pm 0.36 \text{ K}\Omega\text{cm}^2$ .

Fig. 3 shows the changes in the electric resistance with the concentration of the outside solution. At each step, the resistance was measured frequently until a new equilibrium state had been reached. The time interval between two successive measurements was about 10 min. The NaCl solution in the outer chamber always caused a lower electric resistance than the KCl solution of the same concentration.

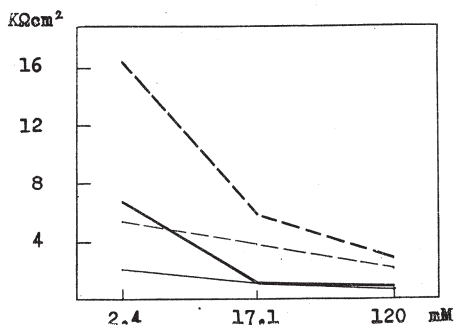


FIG. 3. The electric resistances of the frog skin as functions of the outside NaCl (continuous lines) and KCl (broken lines) concentrations. The inside solution was always Ringer.

2. *Concentration-F.S.P. curve.* Fig. 4 shows the relationship between F.S.P. and the constitution of the solution in the outer chamber. The solution in the inner chamber was Ringer solution throughout the experiment and the solution in the outer chamber was KCl or NaCl solution, concentration of which was changed step by step.

The characteristic maximum of F.S.P. was observed at the concentration of 4–20 mM of NaCl. Using KCl, the increase in F.S.P. with the rise in the concentration up to 4 mM was not so remarkable as in the case of NaCl.

These results coincide with those reported by Hashida (1922), Motokawa (1935), Linderholm (1952) and others.

3. *S.C.C. and F.S.P.* When the inward-facing surface of the frog skin was remained in contact with Ringer solution and the outside Ringer solution was

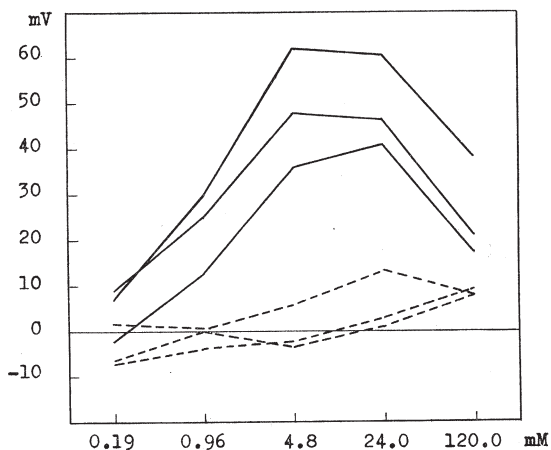


FIG. 4. The potential differences across the frog skin as functions of the outside NaCl (continuous lines) and KCl (broken lines) concentrations. The inside solution was always Ringer solution.

replaced with the isotonic KCl solution, F.S.P. and S.C.C. diminished and kept the smaller value after some drift. The magnitude of F.S.P. after this replacement of the solution ranged from ca. 10 to 30 mV, and that of S.C.C. from ca. 10 to 20  $\mu\text{A}/\text{cm}^2$ . When the outside solution was replaced again with Ringer solution, there appeared once a higher value of F.S.P. (and S.C.C.), declining gradually to the equilibrium within one hour or more. These results are illustrated in Fig. 5.

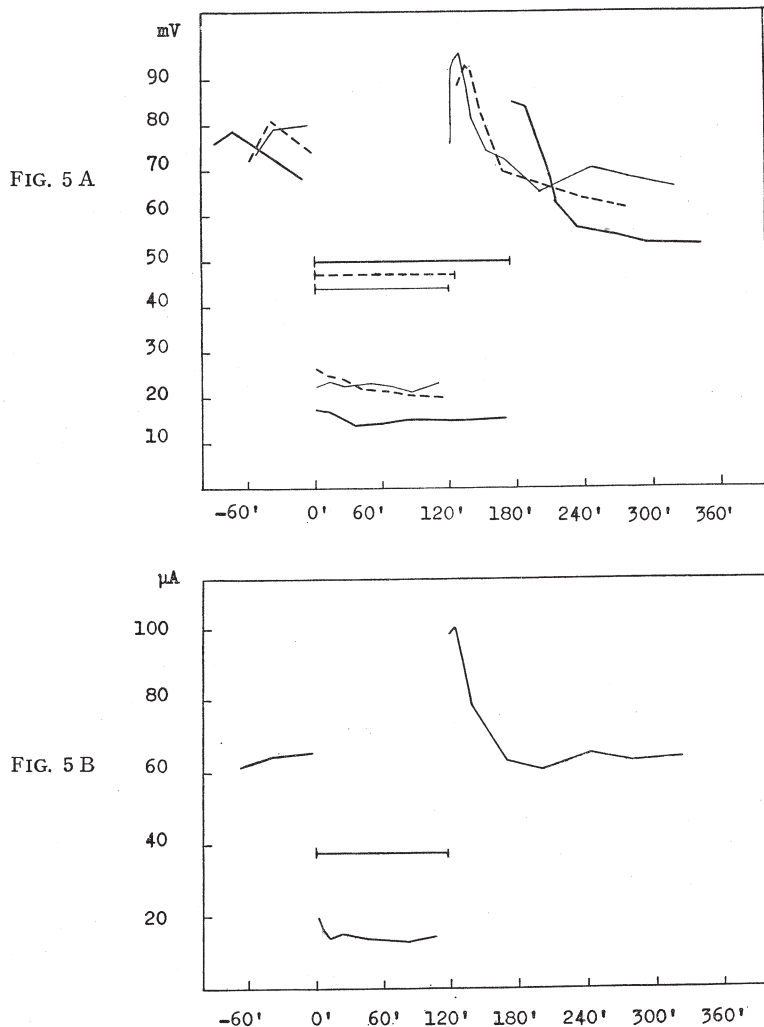


FIG. 5. Effects of the substitution of the outside bathing solution on F.S.P. (A) and S.C.C. (B).

Horizontal lines denote the periods in which the frog skin was in contact with KCl solution on the outside and Ringer on the inside. In other periods the frog skin was in contact with Ringer solution on both sides.

When the skin was treated with  $1 \cdot 10^{-5}$  M of ouabain, a specific inhibitor of active transport, for about two hours, both the stationary F.S.P. (and S.C.C.) and the transitional higher F.S.P. (and S.C.C.) did not appear (cf. Fig. 6).

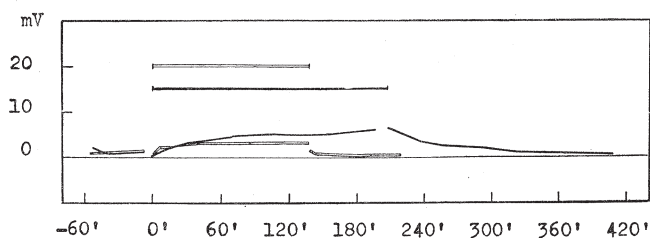


FIG. 6. All conditions were the same as Fig. 5. A except that the skin had been treated with ouabain.

When Ringer solution in the inner chamber is replaced with the isotonic KCl solution, F.S.P. (and S.C.C.) diminished or disappeared after some drift. After a short period the substitute, inside KCl, was again replaced with Ringer, then F.S.P. (and S.C.C.) recovered the initial level. On the other hand, when the contact with KCl lasted for more than one hour, the recovery of F.S.P. was only partial after the exchange of the solution. These results are illustrated in Fig. 7.

By replacement of Ringer solution with the isotonic KCl solution on both sides, F.S.P. (and S.C.C.) almost disappeared or slightly reversed. The following replacement with Ringer solution in both chambers within a short period caused higher F.S.P. (and S.C.C.) than that after the equilibrium was reached. If immersion of the skin in KCl solution was kept for more than one hour, F.S.P. (and S.C.C.) was decreased after a temporary rise to nearly 0 mV within one hour and recovered usually very slowly to near the initial level. These are illustrated in Fig. 8.

#### DISCUSSION

Ussing's assumption that the outside-facing membrane of the frog skin has a high permeability to sodium and the inside-facing membrane has a high permeability to potassium is consistent with our results on the electric resistance of frog skin.

The relation between the frog skin potential and the constitution of the outer solution makes it possible to analyse the ionic permeabilities of the outside facing membrane.

Assuming that the membrane potential may be approximated by the equation derived by Hodgkin and Katz (1949), the frog skin potential can be approximated as follows,

$$E = \frac{RT}{F} \ln \frac{P_{Na}(Na)_o + P_K(K)_o + P_{Cl}(Cl)_o}{P_{Na}(Na)_c + P_K(K)_c + P_{Cl}(Cl)_o} + \frac{RT}{F} \ln \frac{P_{Na}'(Na)_c + P_K'(K)_c + P_{Cl}'(Cl)_i}{P_{Na}'(Na)_i + P_K'(K)_i + P_{Cl}'(Cl)_c}$$



where  $P_{Na}$ ,  $P_K$  and  $P_{Cl}$  are the permeabilities of the outer membrane to the respective ions,  $P'_{Na}$ ,  $P'_K$  and  $P'_{Cl}$  are those of the inner membrane, while  $( )_i$ ,  $( )_o$ , and  $( )_c$  denote the concentrations of the respective ions enclosed in brackets, in the inside, in the outside bathing solution, and in the cell, respectively.

There are nine unknown ( $P_{Na}$ ,  $P_K$ ,  $P_{Cl}$ ,  $P'_{Na}$ ,  $P'_K$ ,  $P'_{Cl}$  and  $(Na)_o$ ,  $(K)_c$ ,  $(Cl)_c$ ) in this equation. As it is difficult to get the concentration-potential curve concerning with the inside surface of the frog skin, it is hard to get the ionic

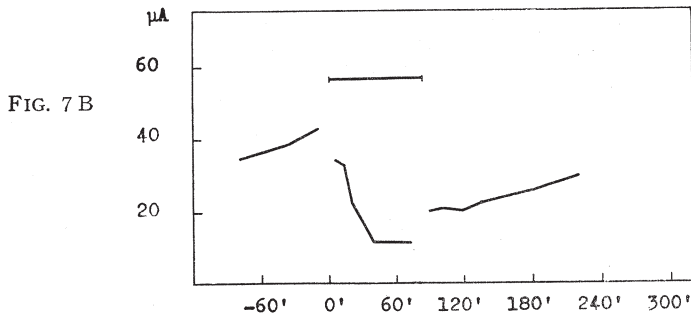
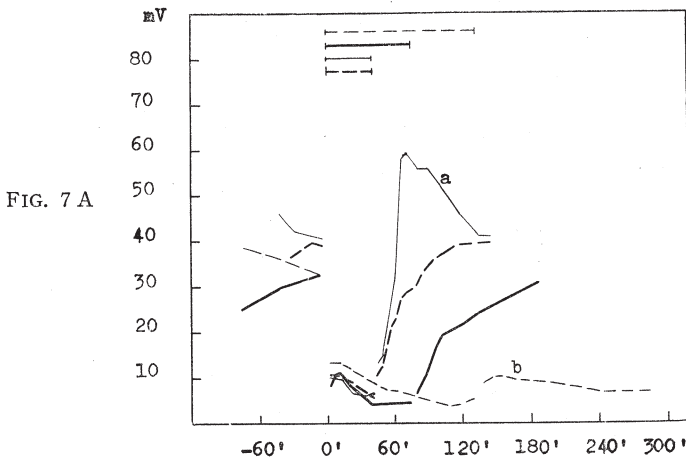


FIG. 7. All conditions were the same as Fig. 5. except that the frog skin was in contact with Ringer solution on the outside and KCl solution on the inside during the periods denoted with horizontal lines. Two exceptional cases are illustrated too;

- (a) There appeared once a high F.S.P.. The inhibition of the active transport has been considered to be incomplete.
- (b) The F.S.P. did not recover, because the inside surface of the frog skin was in contact with KCl too long.

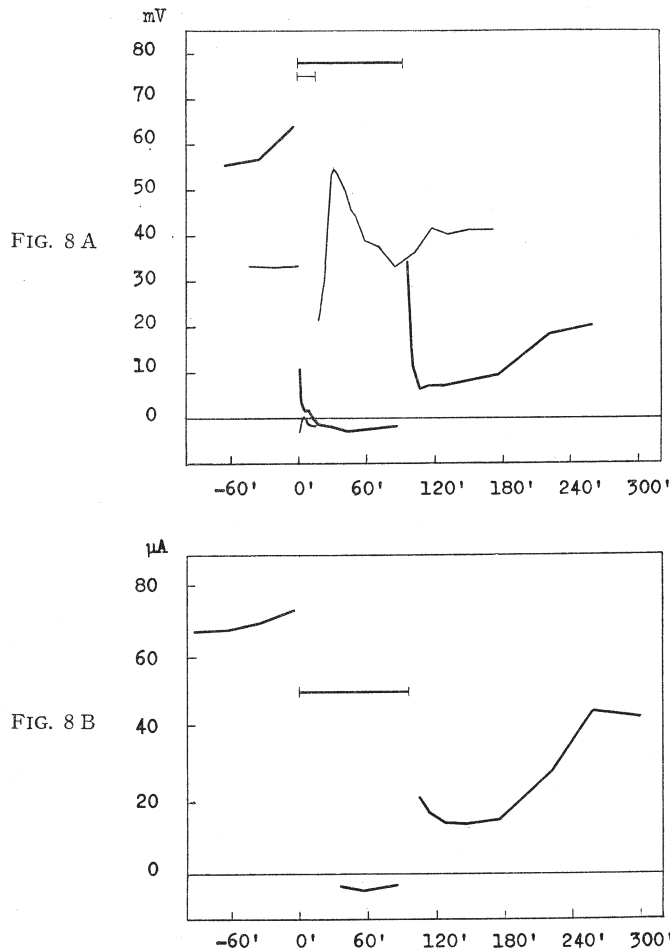


FIG. 8. All conditions were the same as Fig. 5. except that the frog skin was in contact with KCl solution on both sides during the period denoted with horizontal lines.

permeabilities of the inner membrane. Moreover the values of the unknown should vary in some extent according to the constitutions of the extracellular solutions. Therefore it is unavoidable that some degree of freedom takes place with the determination of the values of the unknown. An example of a set of the values tentatively obtained has been shown in Table 1, where  $P_{Na}$  is larger than  $P_K$ ,  $P'_K$  larger than  $P'_{Na}$ , the cellular K concentration is high and the cellular Cl concentration is low. Theoretical curves calculated from these values are shown in Fig. 9. These curves resemble comparatively well to the experimental curves in Fig. 4, excepting the concentration of NaCl solution of the outer chamber above 5 mM. It is noteworthy that K permeability of the outer membrane cannot be zero in spite of the existence of some degree of



TABLE 1. A Set of the Values, tentatively obtained, of the Ionic Permeabilities of the Outer Membrane, Inner Membrane and the Ionic Concentrations in the cell

| Permeability to           | Na | K   | Cl     |
|---------------------------|----|-----|--------|
| of the outer membrane     | 15 | 1   | 9      |
| of the inner membrane     | 1  | 20  | 13     |
| Cellular concentration of | 1  | 110 | 0.4 mM |

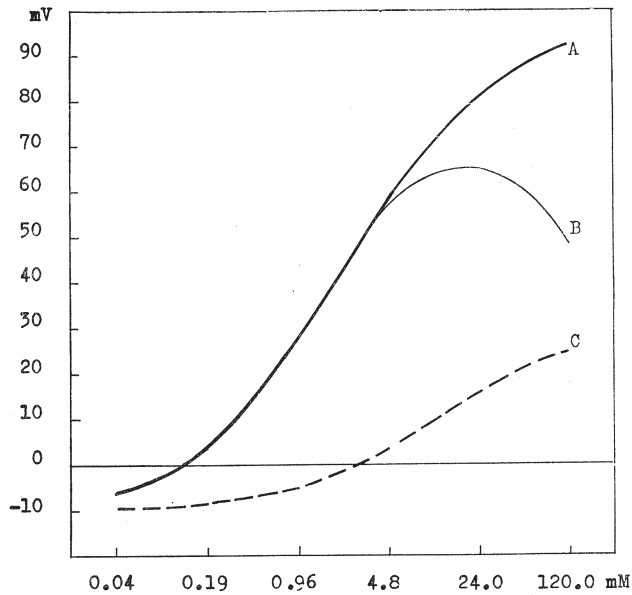


FIG. 9. The potential differences across the frog skin as functions of the outside NaCl (A, B) and KCl (C) concentrations.

(A, C) : Theoretically obtained by using a set of values in Table 1.  
 (B) : Experimentally obtained.

freedom accompanied by the determination of a set of the values. This fact and the high concentration of K ions in the cell seems to be able to explain Steinbach's observation that K ions can be transported across the frog skin and be more easily transported from the inside to the outside than from the outside to the inside (Steinbach, 1937).

As the concentration of NaCl solution in the outer chamber becomes higher, the experimental curve begins to deviate downward from the theoretical curve passing through a maximum. Many researchers have tried to explain this fact (Hashida 1922, Motokawa 1935, Linderholm 1952). The two membrane theory throws a new light on the elucidation of this phenomenon.

In the situation in which the concentration of the outside solution is low, the ionic constitution of the intracellular fluid may be considered to be constant, as the situation is not so different from the physiological condition

for the frog skin. Under a situation like this, it is considered that the quantity of ions which flow into the cell must be small and can be pumped out completely by the active transport, which has been assumed to be a forced exchange of Na ions from the cells against K ions from the inner solution (HUF, Doss and Wills 1957, Johnsen, Ussing and Zerahn 1958).

As the concentration of NaCl solution in the outer chamber become higher, the influx of NaCl into the cell from the outside must increase, exceeding the capacity of the active mechanism to pump them out. Then the intracellular concentrations of Na ions and Cl ions increase and that of K ions decreases. These changes in ionic concentrations act to diminish the frog skin potential.

When the concentration of K ions in the outer solution is increased the influx of KCl does not increase so much as in the case of NaCl, not affecting the ionic constitution of the cellular fluid, because the permeability of the outer membrane to K ions is small. It therefore does not affect the frog skin potential remarkably. Those conditions relating potential changes are illustrated schematically in Fig. 10.

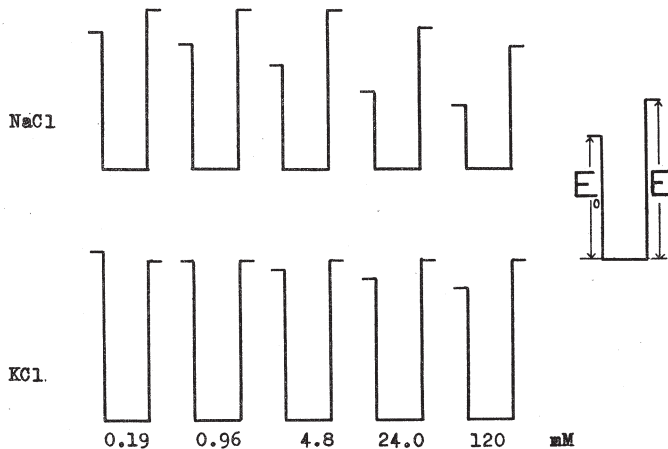


FIG. 10. Schematic illustration of the generation of the frog skin potential (see text).

$E_0$  : Membrane potential across the outer membrane.

$E_i$  : Membrane potential across the inner membrane.

Upper series: The outside solution was NaCl.

Lower series: The outside solution was KCl.

The short-circuit current which is generated when the outward-facing surface of the frog skin is in contact with isotonic KCl solution and the inward-facing surface with Ringer solution can be explained as follows: Under such a situation the intracellular concentration of Na ions is kept low by the effect of the active transport. Though Cl ions are in equilibrium at the short-circuited state, K ions and Na ions are facing to each other across

the inner cell membrane, which is more permeable to K ions than to Na ions. Then passive diffusion of K ions through the inner membrane is greater than that of Na ions in the opposite direction. S.C.C. from the outside to the inside is generated as the result of it. The generation of F.S.P. can be similarly explained. When the active process is interrupted by ouabain, the intracellular concentration of Na ions is increased and that of K ions is decreased. S.C.C. (and F.S.P.) diminishes accordingly.

When both chambers are filled with the isotonic KCl solutions, the intracellular Na ions are replaced with K ions so that K and Cl ions should be distributed according to the Donnan's equilibrium if K and Cl ions is not actively transported. As the result, F.S.P. (and S.C.C.) disappears. But it seems to be possible that the slight reversion of the potential difference is caused by active potassium transport decoupled from active sodium transport.

It is noteworthy that high F.S.P. (and S.C.C.) appears (Fig. 5, 8) after KCl solution in the outer chamber or the both chambers are again replaced with Ringer solutions. It can be explained as follows: The intracellular K ion level has been elevated and the intracellular Na ion level has been suppressed in such a situation. Though the extracellular K ions is removed by the replacement, K ions yet remain higher and Na ions lower in the cell. This situation causes high F.S.P. (and S.C.C.). As the constitution of the cellular fluid approaches an equilibrium after the inverse replacement, F.S.P. (and S.C.C.) also approaches the equilibrium value.

The diminution or the disappearance of F.S.P. (and S.C.C.) after the replacement of Ringer in the inner chamber with the isotonic KCl solution can be partly explained by Ussing's hypothesis. Fukuda's results that the removal of K ions from the inner solution causes a temporary elevation in F.S.P. and the addition of K ions to it causes the diminution of F.S.P. (Fukuda, 1942) can be explained similarly.

However, it is possible that another factor takes part in the diminution of F.S.P. and (S.C.C.) after the substitution of the inner KCl solution for Ringer solution. The inverse replacement of the inner KCl solution with Ringer causes only low F.S.P. (and S.C.C.) as shown in Fig. 7, when the former substitution of KCl solution lasts for more than one hour. So it is considered that the concentration of the cellular K ions has been lowered and the concentration of the cellular Na ions has been elevated. Such a situation can be considered to be due to interruption of active transports. Therefore it may be concluded that the forced exchange is inhibited when the ionic circumstance is not appropriate, as in the case of erythrocytes reported by Post *et al.* (1960).

#### SUMMARY

- 1) The electric resistance of the frog skin while Ringer solution was placed on both sides of the skin was compared with the electric resistance while Ringer solution was placed on the outside and KCl solution on the inside. The result was that the resistance of the former was greater than that of the latter.

When the resistance of the former is compared with that in case of KCl on the outside and Ringer on the inside, the former was smaller.

These results are easily explained from the Ussing's assumption that the outer membrane has a larger permeability to Na ions than to K ions and the inner membrane a smaller permeability to Na ions than to K ions.

2) The relationship between the electric potential and the constitution of the outside solution was applied to analyse the ionic permeabilities of the membranes. The results tentatively obtained are illustrated in Table 1.

Characteristic Maximum in the outside NaCl concentration-potential curve can be considered as the result of an increase of the cellular NaCl concentration.

3) Assuming that the cellular concentration of K ions is always high as the effect of the active process, the frog skin potential when it was in contact with KCl on one or both sides can also be explained as the sum of the diffusion potentials across the inner membrane and that across the outer membrane. The short-circuit current in the same situation can be explained similarly.

The small potential difference in the case of KCl solutions on both sides is interpreted as the result of decoupling of the active K transport from the active Na transport.

4) Transitional phenomena following the replacement of immersing solutions can be explained assuming that the cellular ionic constitution has a tendency to shift toward the extracellular ionic constitutions.

It is necessary to postulate that the elimination of Na ions from the inner solution causes an interruption of the active transport.

In conclusion, these electrical natures of the frog skin can be explained from the viewpoint of Ussing's "Two membrane theory."

The author wishes to thank Professor R. Ito for his helpful discussions and advices.

#### REFERENCE

1. ENGBAER, L. AND T. HOSHIKO: *Acta Physiol. Scand.* **39**: 348, 1957.
2. FUKUDA, T. *Jap. J. Med. Sci. Part III Biophys.* **8**: 123, 1942.
3. HASHIDA, K. *J. Biochem.* **1**: 21, 1922.
4. HODGKIN, A. L. AND B. KATZ. *J. Physiol.* **108**: 37, 1949.
5. HUF, E. G., DOSS, NORMA S. AND WILLS, JOYCE, P. *J. G. Physiol.* **41**: 397, 1957.
6. JOHNSEN, V. K., USSING, H. H. AND K.ZERAHN. *Acta Physiol. Scand.* **42**: 298, 1958.
7. LINDERHOLM, H. *Acta Physiol. Scand.* **27**: suppl, 97, 1952.
8. LINDERHOLM, H. *Acta Physiol. Scand.* **31**: 36, 1954.
9. MOTOKAWA, K. *Jap. J. Med. Sci. Biophys.* **3**: 203, 1935.
10. POST, R. L., C. R. MERRIT, C. R. KINSOLVING AND C. D. ALBRIGHT. *J. Biol. Chem.* **235**: 1796, 1960.
11. STEINBACH, H. B. *J. Cell. Comp. Physiol.* **10**: 51, 1937.
12. USSING, H. H. *Acta Physiol. Scand.* **19**: 43, 1949.
13. USSING, H. H. AND K. ZERAHN. *Acta Physiol. Scand.* **23**: 110, 1951.
14. USSING, H. H. *J. G. Physiol.* **43**: 135, 1960.