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Electrical response of frog skin epidermis to sodium ions

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ELECTRICAL RESPONSE OF
FROG SKIN EPIDERMIS
TO SODIUM IONS

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ELECTRICAL RESPONSE OF
FROG SKIN EPIDERMIS
TO SODIUM IONS

by

James H. Martin, III

A thesis submitted to the Faculty of the Graduate
School of the University of Richmond in partial
fulfillment of the requirements for the Degree of
Master of Science.

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ABSTRACT ·

Presently, the theory is held that the total frog skin potential (P.D.) is generated within the epidermis at two borders, the "outer border" and the "inner border," which are said to be specifically permeable to Na^+ and K^+ , respectively. This thesis concerns itself only with the electrical response of the "outer border" to varying Na^+ concentrations in the solutions at the epidermis, $[\text{Na}^+]_o$. Contrary to expectation from the Nernst equation, the P.D. changes by only 17 to 35 mV, instead of theoretically 58 mV upon a 10 fold change in $[\text{Na}^+]_o$. This paper shows that it is very unlikely that the discrepancy between theory and experiment results from the participation of movement of K^+ , H^+ , and SO_4^- across the "outer border" which indeed, seems to be specifically permeable to Na^+ . Results for epinephrine treated skins suggest that this Na^+ specificity is completely lost. A theoretical treatment of the mechanism of generation of the "outer border" skin P.D., presented in this thesis, shows that the difference between theory and experiment can be explained if two factors are added to the concept that the "outer border" skin P.D. is a Na^+ diffusion potential. These factors are: 1) Continuous active transport of Na^+ across the skin, and 2) Diffusion delay within the epidermis in the layers in front of the "outer border." Taking these two factors into account, a modified Nernst equation was derived to show the dependence of the skin P.D. on varying $[\text{Na}^+]_o$. It is given by Equation (18) in the List of

Equations. A test for this equation shows that it adequately describes the response of the epidermis of the frog skin to varying Na^+ concentration at the epidermal side of the skin.

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INTRODUCTION

A. HISTORICAL REVIEW

Bio-electricity has been known since ancient times. In the Egyptian writings there are references to Malopterus. The Romans gave the name "Torpedo" to the electric ray. By the end of the eighteenth century it was suggested that the shock received from electric fish was similar to the electrostatic discharge received from the Leyden jar. In 1786 Galvani obtained evidence that electric currents were present in nerves as well as muscles. In 1848 Emil Du Bois-Reymond published a book, Untersuchungen über Thierische Elektrizität (Researches on Animal Electricity), with methods of measuring various bio-potentials. The frog skin potential was first mentioned in this book.

Today bio-potentials are well known and are by no means confined to laboratory and research studies. Physicians use recordings of the potentials developed by the brain (EEG) or heart (EKG) to find certain anomalies which will aid in diagnosis and treatment of diseases. Indeed, irritability, a characteristic of all living organisms, always in some way involves electric potentials. Most of these potentials are developed by ionic equilibria and active transport across membranes. Individual cells maintain a potential difference between their internal and external environments, and for this reason neurons and the large cells of certain algae such as Valonia and Mitella are used extensively in membrane

potential research.

However, because of their small size, individual cells offer many technical difficulties. Frog skin, it would appear, has eliminated the problem of small size. Du Bois-Reymond discovered that the isolated frog skin maintained a potential difference across itself, the outer epidermal surface being negative with respect of its corium surface. Huf (1935, 1936) discovered that the isolated frog skin could actively move chloride, as NaCl, from a Ringer's solution bathing the epidermal side to a Ringer's solution bathing the corium side. Ussing (1951) advanced the hypothesis that frog skin could transport sodium chloride against both an electric potential and a concentration gradient. Ussing suggested that sodium was transported and that chloride moved passively with it, thus preserving charge neutrality. As sodium is actively transported across the skin, a potential gradient is established, and chloride moves passively following the electrical gradient. In order to test this hypothesis, Ussing and Zerahn (1951) devised the short circuit technique to eliminate the potential gradient. An isolated frog skin was mounted between two chambers containing Ringer's solution of the same concentration. The developed skin potential was monitored. A current was applied in such a direction as to reduce the potential across the skin to zero. This is called "short circuiting" the skin. With this arrangement, the only transport which could occur would be the result of an active process. From the results of this experiment,

Ussing and Zerahn concluded that only sodium was transported, and that in the "open skin," the sodium transport mechanism and passive movement of the anion was responsible for the skin EMF.

Most investigators believe that there are two electrogenic layers in the frog skin. Steinbach (1933) gave evidence that there are at least two layers involved in the production of the skin potential. Assuming the existence of an "outer border" (epithelium facing layer) and an "inner border" (corium facing layer), it has been shown by Fukuda (1942, 1944) that the outer layer requires the presence of sodium but not potassium. Fukuda suggested that the "outer border" was preferentially permeable to sodium and that the "inner border" was preferentially permeable to potassium. Koefoed-Johnsen and Ussing (1958) proposed a model that emphasized these preferential permeabilities. When they eliminated anion penetration by replacing sodium chloride with sodium sulfate, they found that the skin potential changed by almost 58 mV for a ten fold change in the outside sodium or inside potassium concentration. Therefore, Koefoed-Johnsen and Ussing regarded the total skin potential to be the sum of the two Nernst diffusion potentials of sodium at the "outer border" and potassium at the "inner border." Other workers have tried to confirm the response at the "outer border" but have not found the theoretical 58 mV response. A change of 35 mV for a ten fold change in concentration has been reported by Lindley and Hoshiko (1964), by Winn et al. (1964),

and by Cereijido and Curran (1965).

In order to determine the actual number of barriers or potential steps across frog skin, several investigators have employed the micro-puncture technique for micro-potential measurements. Ottoson et al. (1953) was the first to employ the technique. Others (Engbaeck and Hoshiko, 1967; Scheer, 1960) followed, and the latest to publish their results, Chowhury and Snell (1965), are the only investigators to obtain a continuous potential change across the frog skin. All other workers report two, and sometimes three or more steps.

The exact location of the "borders" is unknown, but it is certain that they lie in the epidermis. The "inner border" appears to lie at the dermo-epidermal junction, and the "outer border" may be the outward facing cell membranes of the stratum germinativum (Figure 1).

B. HISTOLOGY OF THE FROG SKIN

The epidermis of adult Rana pipiens has been studied in detail by histological, histochemical, and electron microscopy methods. Figure 2 is a photomicrograph of the belly skin of Rana pipiens in cross section. It shows the dermis (corium) and the associated glands. The dermis will not be discussed here, since it plays little or no part in the development of the skin potential. Figures 1 and 2 show the general organization of the abdominal skin of the frog. The epidermis is a stratified epithelium with a few of its layers forming the stratum corneum, which is composed

of partially cornified squamous cells. Beneath this are one to three layers of cuboidal and polyhedral cells which are sometimes differentiated into stratum granulosum and stratum spinosum. There is a basal layer of cuboidal and columnar cells, the stratum germinativum. Electron micrographs show a definite basement membrane at the dermo-epidermal junction. The junctions between adjacent cells in the stratum corneum show the outer leaflets of the membranes fused into a single dense band 30-40 Å thick, and the total distance across the two fused membranes measures 170-180 Å.

In the stratum granulosum the component membranes are smaller, and the junctions measure about 120-140 Å across the two fused membranes. There is extensive and complex interdigitation between the cells of the stratum germinativum.

C. THEORY OF FROG SKIN BIO-POTENTIALS

One widely held concept about the origin of bio-potentials is that cell membranes behave as ion sieves. They can bring about noticeable separation of ionic charges across the membrane. Thus, for the special case of a membrane specifically permeable to sodium, one may observe a potential difference, V , across the membrane which can be calculated by the Nernst equation:

$$V = \frac{RT}{nF} \ln \frac{C_1}{C_2} \quad (1)$$

where R is the gas constant taken as 8.3 joules per degree

per mole; T is the absolute temperature; n is the valency of the ion species with the appropriate sign; F is Faraday's constant (96,500 coulombs per gram equivalent); and \ln is the natural logarithm ($2.3 \times \log_{10}$). If C_1 and C_2 are the Na^+ ion concentrations on the two sides of the membrane, and the absolute room temperature is 297 K, Equation (1) can be written:

$$V = 58 \log \frac{[\text{Na}^+]_1}{[\text{Na}^+]_2} \quad (2)$$

Thus for a ten fold difference in sodium concentration across the membrane, $V = 58$ mV. If the "outer border" of frog skin represents the outward oriented cell membranes of the stratum germinativum and if the cell membranes behave like an ideal sodium selective membrane, the the potential difference (V_{ob}) across this border may be written:

$$V_{\text{ob}} = 58 \log \frac{[\text{Na}^+]_o}{[\text{Na}^+]_c} \quad (3)$$

where $[\text{Na}^+]_o$ is the sodium ion concentration of the solution at the epidermal side, and $[\text{Na}^+]_c$ is the intracellular sodium ion concentration, which will be taken as 10 $\mu\text{Eq/ml}$ (Andersen and Zerahn, 1963). If $[\text{Na}^+]_o$ equals 100 $\mu\text{Eq/ml}$, then $V_{\text{ob}} = 58$ mV.

As will be shown in the following portions of this thesis, which concerns itself exclusively with the electrical

events at the "outer border," one rarely observes the theoretical value. The highest value for V_{ob} generally observed is only 35 mV. At present, the reason for this discrepancy is unknown. It must be pointed out, however, that the assumption made in the above calculation is that the "outer border" is permeable only to sodium ions. One may question the validity of this assumption, since under the experimental conditions which prevail, ions other than sodium ions are present when V_{ob} measurements are made. The predominant anions are chloride, when chloride Ringer's is used, and sulfate, when sulfate Ringer's is used. Potassium and hydrogen are also present. If the "outer border" is bathed in sulfate Ringer's and is permeable to all of these ions, a potential difference will be generated which may be calculated from the Hodgkin-Katz equation (1949):

$$V_{ob} = \frac{RT}{F} \ln \frac{P_{Na} [Na^+]_o + P_K [K^+]_o + P_H [H^+]_o + P_{SO_4} \sqrt{[SO_4^{--}]_c}}{P_{Na} [Na^+]_c + P_K [K^+]_c + P_H [H^+]_c + P_{SO_4} \sqrt{[SO_4^{--}]_o}} \quad (4)$$

This equation states that the membrane potential results from the movement across the membrane of all ions, each of which makes a contribution according to its concentration and its permeability coefficients. The P's in Equation (4) are the various ion permeability coefficients. On this basis, it could be readily explained why one finds only a potential difference of 35 mV when actual measurements on V_{ob} are taken, instead of the 58 mV predicted by the Nernst equation (3). It is difficult to prove whether or not this

is the correct explanation for the generation of V_{Ob} since knowledge about the intracellular ion concentration and the permeability coefficients is rather difficult to obtain.

It can be reasoned, however, that the application of the Hodgkin-Katz equation to the "outer border" of frog skin does not explain why V_{Ob} is lowered to 35 mV, instead of being 58 mV. The reasons are as follows:

1) When sulfate Ringer's is used, one can be fairly certain that movement of anions is excluded from participation in the generation of V_{Ob} . In contrast to chloride ions (which are present in ordinary Ringer's) membranes are generally known to be impermeable to sulfate ions. Overall sulfate permeability studies show a low permeability coefficient for sulfate (Table 1).

Ignoring for a moment the possible participation of potassium and hydrogen ions (justification of which will be given below), and recognizing that at the beginning of an experiment with sulfate Ringer's, the intracellular sulfate concentration must be near zero, one can write:

$$V_{Ob} = 58 \log \frac{P_{Na} [Na^+]_o}{P_{Na} [Na^+]_c + P_{SO_4} \sqrt{[SO_4^{=}]_o}} \quad (5)$$

Taking for $[Na^+]_o$ the value often used in experimental studies, namely 110 μ Eq/ml, and for $[Na^+]_c$ the value of 10 μ Eq/ml, one can calculate $V_{Ob} = 60.4$ mV, in the case of complete impermeability of the "outer border" to sulfate ions. On the other hand, if $P_{SO_4} = 0.4 \times 10^{-8}$ cm/sec

(Table 1) and $P_{Na} = 0.4 \times 10^{-6}$ cm/sec (Table 1), one obtains for Equation (5) a value of V_{Ob} which is only 0.04% less than 60.4 mV, showing the small contribution that sulfate ion movement would make to V_{Ob} . Again using Equation (5), one can calculate that a value of $P_{SO_4} = 0.9 \times 10^{-6}$ cm/sec could satisfy the experimental finding of $V_{Ob} = 35$ mV, instead of 58 mV. In other words, only if the permeability of the "outer border" to sulfate ions was on the order of the permeability to sodium ions could the experimental data be explained by the Hodgkin-Katz equation. Such a high P_{SO_4} value is quite unlikely on the basis of the work of MacRobbie and Ussing (1961) who showed from osmotic studies of frog skin epidermis that "the outward facing membrane is permeable to Na^+ and Cl^- , but not to $SO_4^{=}$."

2) Returning to Equation (4), it is also highly unlikely that movement of K^+ ions across the "outer border" makes a significant contribution to V_{Ob} . This follows from the very low P_K values (Winn et al., 1964) of less than 1×10^{-6} cm/sec (close to 1×10^{-7} cm/sec). Chowdhury and Snell (1965) have estimated P_K to be between 1×10^{-8} and 1×10^{-7} cm/sec.

3) As for the possible contribution of H^+ ions to V_{Ob} , it must be pointed out that the absolute concentration of this ion on both sides of the "outer border" is extremely low, on the order of 10^{-7} μ Eq/ml. Equation (4) predicts an increase in V_{Ob} with increasing $[H^+]_o$. This is contrary to what is found.

From this analysis, the conclusion is reached that the predominating ion which determines the value of V_{ob} is the sodium ion, and that Equation (3) should indeed hold. The reason for the discrepancy between the predicted value of 58 mV (for a ten fold change in sodium concentration) and the experimentally found value of 35 mV or less, therefore, must lie outside of the consideration that ions other than sodium ions may play a role. Therefore, it was the purpose of this research to examine whether other factors, namely sodium diffusion delay within the epidermis and active transport of sodium across the skin, could account for the discrepancy between experiment and theory on the electrical response of the "outer border" of the frog skin epidermis to variation in the sodium ion concentration.

MATERIALS AND METHODS

A. DISSECTION AND MOUNTING OF SKIN MEMBRANE

The living belly skin of Rana pipiens was used for all experiments. The frogs were obtained commercially from Steinhilber, Oshkosh, Wisconsin. They were kept in a tank supplied with running tap water. They were not fed and were used within 14 days after receipt.

The frogs were decerebrated with scissors (Figure 3) and the remaining portions of the brain and spinal cord were destroyed with a dissection needle. As rapidly as possible the skin over the xiphisternum was punctured and cut laterally (Figure 4) both left and right, cutting the cutaneous arteries at the same time. The cut was continued caudally on both sides of the body in the pigmented area of the skin. Just ventral to the cloaca, the incisions were joined with a final cut across the pelvic region.

The skin is held to the body by fascia running along the ventro-lateral portion of the body and meeting at the point of the final incision across the ventral pelvic region. By pulling the skin away from the body wall musculature, the skin-muscle junction can be easily seen as a transparent connecting zone of fascia. By cutting these transparent fascia, the skin section was removed from the body without any adhering muscle.

The skin was spread on a porcelain plate and carefully blotted free of excess mucus, blood, and moisture. The

circular piece of skin (Figure 5) was mounted as a membrane separating two chambers of a lucite cell (Figure 6 and 7). It was placed across the open end of one of the cell chambers, the other chamber placed on top (Figure 6), and the cell was then completely assembled and tightened. Each cell was then placed in the cell positioner over a set of magnetic stirrers (Figure 8) for continuously mixing the contents of each chamber. The area of the circular skin membrane between the two chambers was 7.25 cm^2 . The chamber on the epidermal side of the skin is referred to as the outer chamber. The chamber on the dermal side of the skin is referred to as the inner chamber. Each chamber was completely filled with 25 ml of the desired Ringer's solution. The stirring bars for the magnetic stirrers were dropped into each chamber, and the stirrers were turned on.

B. DESCRIPTION OF INSTRUMENTS

All potential difference (P.D.) experiments were conducted using the same instrumental arrangement and equipment. Potential measurements were made with a Keithley Model 600A Multipurpose Electrometer. The electrodes used were Radiometer Calomel electrodes, Type K401.

Potential difference measurements were made by lowering the electrodes into the cells through the holes in top of each chamber (Figure 7). The apparatus could accommodate two cells, and thus two experiments could be run simultaneously. Figure 8 shows the apparatus. The electrode selector switch was turned to the right, and the P.D. of the right

hand cell was measured. The electrode selector switch was then turned to the left, and the P.D. of the left hand cell was measured.

C. POTENTIOMETRIC STUDIES TO TEST THE RESPONSE OF THE EPIDERMIS TO CHANGING $[Na^+]_o$.

Stock solutions of 0.5 M Na_2SO_4/l , 0.5 M K_2SO_4/l , and 0.5 M THAM/l (tris hydroxymethyl aminomethane buffer) were prepared. Using these stock solutions, four types of Ringer's solutions were prepared (Table 2). The sodium and potassium concentrations were varied, the molar sum of the sodium and potassium always being 120 mM/l. The pH of each Ringer's solution was adjusted to 8.0 with H_2SO_4 using a Beckman Model G pH meter as described in Table 2. Before every experiment, oxygen was bubbled through each Ringer's solution for three minutes.

Experiments were conducted in pairs. The skins were mounted between the chambers of each cell as previously described. Ringer's solution no. 1 (110 mM Na/l--10 mM K/l) was added to both inside and outside chambers of each cell. The cells were placed in the cell positioner and allowed to equilibrate for one hour. At the end of this hour, the P.D. was measured and recorded. The Ringer's solution was removed from both chambers of each cell. The outside chambers were rinsed and filled with Ringer's solution no. 2 (90 mM Na/l--30 mM K/l). The inside chambers were rinsed and filled with fresh Ringer's solution no. 1. At the end of fifteen minutes, the P.D. was measured and recorded. The solutions

were removed from both the inside and outside of each cell. The outside chambers were rinsed and filled with Ringer's solution no. 3 (60 mM Na/l--60 mM K/l), and the inside chambers were rinsed and filled with Ringer's solution no. 1. At the end of fifteen minutes, the P.D. was measured and recorded. The cells were emptied as before. Then the outside chambers were rinsed and filled with Ringer's solution no. 4 (10 mM Na/l--110 mM K/l), and the inside chambers were rinsed and filled with Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded. The cells were emptied and both inside and outside chambers were rinsed and filled with Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded.

D. POTENTIOMETRIC STUDIES ON THE EPIDERMIS OF FROG SKIN IN ISETHIONATE RINGER'S SOLUTION

Stock solutions of 1 M sodium isethionate/l ($\text{HO-CH}_2\text{-CH}_2\text{-SO}_3\text{Na}$, obtained from Eastman Kodak Co.), 0.5 M Na_2SO_4 /l, 0.5 M K_2SO_4 /l, and 0.5 M THAM/l were prepared. Using these stock solutions, three Ringer's solutions (no. 1, no. 5, and no. 6) were prepared (Table 2). Before each pair of experiments, oxygen was bubbled through the Ringer's solutions for three minutes. Skins were prepared and mounted as before.

Sulfate Ringer's solution no. 1 (110 mM Na/l--10 mM K/l) was placed in each chamber, and the cells were put in the cell positioner. The stirring bars were dropped into each chamber and adjusted to mixing speed. After one hour, the P.D. was measured and recorded. The cells were emptied, and

the outer chambers were rinsed and filled with sulfate Ringer's solution no. 5 (120 mM Na/l). The inner chambers were rinsed and filled with sulfate Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded. The outer chambers were rinsed and filled with isethionate Ringer's solution no. 6 (120 mM Na-isethionate/l), and the inner chambers were rinsed and filled with sulfate Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded. The chambers were emptied, rinsed and filled with sulfate Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded.

E. THE OVER-ALL PERMEABILITY OF FROG SKIN TO S^{35} LABELED
SULFATE

Using the 0.5 M/l stock solutions of Na_2SO_4 , K_2SO_4 , and the stock solution of THAM, one liter of sulfate Ringer's solution no. 1 (110 mM Na/l--10 mM K/l) was prepared (Table 2). To two 100 ml portions of this Ringer's solution was added $Na_2S^{35}O_4$ in an amount calculated to give 10^4 counts per minute per 0.1 ml. The amount of inactive carrier sodium added to the Ringer's solution was negligibly small.

Skins were mounted, and the outer chambers were filled with Ringer's solution no. 1 containing S^{35} labeled Na_2SO_4 . The inner chambers were filled with Ringer's solution no. 1. After 90 minutes, the P.D. was measured and recorded. A 0.1 ml sample was taken from each chamber, placed in a planchette, and evaporated to dryness.

Count rates were taken for 10 minutes using a gas flow counter (100 $\mu\text{g}/\text{cm}^2$ window) and scaler. Background was taken as the count rate of 0.1 ml sample of fresh, unlabeled Ringer's solution no. 1. The quenching gas was a mixture of isobutane (93%) and helium (7%).

F. THE INFLUENCE OF pH ON THE POTENTIOMETRIC RESPONSE OF THE EPIDERMIS TO $[\text{Na}^+]_o$.

From the stock solutions, the following five Ringer's solutions were prepared (Table 2): Ringer's solution no. 1, pH = 8.0; Ringer's solution no. 5 (120 mM Na/l, pH = 8.0); Ringer's solution no. 7 (120 mM Na/l, pH = 9.0); Ringer's solution no. 8 (120 mM Na/l, pH = 7.0); Ringer's solution no. 9 (120 mM Na/l, pH = 6.0). The pH was adjusted with H_2SO_4 .

The skins were mounted, Ringer's solution no. 1 was added to inner and outer chambers, and the cells placed in the apparatus. After one hour, the P.D. was measured and recorded. The chambers were emptied. The outer chambers were rinsed and filled with Ringer's solution no. 7 (120 mM Na/l, pH = 9.0); the inner chambers were rinsed and filled with Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded. The chambers were emptied, and the outer chambers were rinsed and filled with Ringer's solution no. 5 (120 mM Na/l, pH = 8.0); the inner chambers were rinsed and filled with Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded.

The chambers were emptied. The outer chambers were rinsed and filled with Ringer's solution no. 8 (120 mM Na/l, pH = 7.0); the inner chambers were rinsed and filled with Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded. The chambers were emptied. The outer chambers were rinsed and filled with Ringer's solution no. 9 (120 mM Na/l, pH = 6); the inner chambers were rinsed and filled with Ringer's solution no. 1. After fifteen minutes, the P.D. was measured and recorded.

G. POTENTIOMETRIC STUDIES ON THE EPIDERMIS OF FROG SKIN
OF ANIMALS PRETREATED WITH EPINEPHRINE

L-epinephrine bitartrate (Sigma Chemical Co.) was dissolved in Ringer's solution no. 1 and immediately used. One milligram of epinephrine (0.2 ml of the solution) was injected into the dorsal lymph sac of each frog. The frogs were then placed in a tank supplied with running water. After one hour, the belly skins were removed and mounted as described previously. The same procedure described in Section C was followed to test the response of the skin to changes in sodium concentration.

RESULTS

A. POTENTIOMETRIC STUDIES TO TEST THE RESPONSE OF THE EPIDERMIS TO CHANGING $[Na^+]_o$

Of the thirty experiments conducted, sixteen showed a recovery at the end of a series of testings to within $\pm 10\%$ of the original P.D. Two skins had, respectively, a 12% and a 32% higher skin P.D. compared to the original P.D. The remaining 12 skins showed potentials between 15% and 41% lower than the original P.D. The results obtained on the sixteen experiments with good ($\pm 10\%$) recovery are shown in Table 3. The results were separated into three groups. Male frogs, Group I, gave stronger responses than female frogs, Group II. A greater number of experiments would be needed before stating that the obvious difference seen in the present experiments are sex linked. Because of this uncertainty, the results of the two groups were combined and treated together. The combined data are given in the Group (I + II) column of Table 3. Group III consisted of five additional frogs which responded quite differently from what is most commonly observed. The difference is more clearly seen by comparing Figures 9 and 10. The results obtained in Groups I, II, and I + II are plotted in Figure 9. Figure 10 shows the results obtained in Group III. Figure 9 shows an approximately linear response curve, if P.D. in mV (the dependent variable) is plotted (on the ordinate) against the log of $[Na^+]_o$ (the independent variable on the abscissa). Slope factors, β , for the three

response curves were calculated from:

$$\beta = \frac{V_1 - V_2}{\log \left[\text{Na}^+ \right]_1 / \left[\text{Na}^+ \right]_2} \quad (6)$$

where V_1 and V_2 are skin potentials read from the graph and $\left[\text{Na}^+ \right]_1$ and $\left[\text{Na}^+ \right]_2$ are the associated Na^+ concentrations. Thus, the slope factors of the response lines shown in Figure 9 are:

$$\text{Group I } \delta: \beta = 30$$

$$\text{Group II } \varphi: \beta = 17$$

$$\text{Group I + II } (\delta + \varphi): \beta = 25$$

The response curve of the skins belonging to Group III is a rather complex one. Obviously, the response is not nearly linear. The curve was arbitrarily divided into two portions, as if the response over two regions of $\left[\text{Na}^+ \right]_o$ was approximately linear. The slope factors of the assumed response lines are:

$$\beta = 22 \text{ for } \left[\text{Na}^+ \right]_o \text{ range } 10\text{-}60 \text{ } \mu\text{Eq/ml}$$

$$\beta = 134 \text{ for } \left[\text{Na}^+ \right]_o \text{ range } 60\text{-}110 \text{ } \mu\text{Eq/ml}$$

If the response of the epidermis had followed the Nernst law, β should be 58, and Equation (3) should hold. The dashed line in Figure 9 shows the slope of the theoretical response line.

In the discussion, emphasis will be placed on the slope

factor $\beta = 35$, since this is the highest commonly observed, and the one nearest to the theoretical value, $\beta = 58$. The treatment of data would be the same for the smaller slope factor.

B. POTENTIOMETRIC STUDIES ON THE EPIDERMIS OF FROG SKIN IN ISETHIONATE RINGER'S SOLUTION

If in the potentiometric analysis of the permeability properties of the epidermis, movement of sulfate ions (molecular weight of 96) played a role, then the replacement of the inorganic sulfate by the organic sulfate (isethionate, molecular weight of 125.1) might lead to an increase in skin potential. The results of eight experiments are shown in Table 5. It can be seen that in the presence of isethionate ion, the skin P.D. was not increased, but decreased. Because of the greater molecular weight of isethionate as compared to that of sulfate, an increase in skin P.D. might have been anticipated when sulfate was replaced. This result supports the view held by many authors, that sulfate ion is indeed a rather impermeable ion, and therefore, useful in potentiometric studies designed to evaluate the effect of the cation only. The unexpected P.D.-depressing effect of isethionate has raised the question regarding the mechanism of this effect. It was thought that such a result could be obtained if Na-isethionate were lipid soluble, and hence more readily permeable than inorganic sulfate ions. Some studies were carried out to test for lipid solubility of isethionate. It was insoluble in

benzene and in chloroform. It was soluble, however, in a 1:3 (vol/vol) mixture of ether and ethyl alcohol, and the solubility was 243 mg% when the Na-isethionate was shaken for four hours in the ether-alcohol mixture. It is quite possible, therefore, that the P.D.-depressing effect of isethionate is the result of its solubility in lipids of the Na-selective cell membranes, the "outer border" of the frog skin.

C. THE OVER-ALL PERMEABILITY OF FROG SKIN TO S^{35} LABELED SULFATE

The results of experiments conducted on three frogs is shown in Figure 11. The paired columns showing the activity of the inside (A) and the outside (B) chambers are compared to the background and standard columns. It can be readily seen that in the cases presented, the activity of the inside chambers did not rise above the background level. Any labeled sulfate which passed through the frog skin membrane from the labeled Ringer's on the outside to the unlabeled Ringer's on the inside was in such a small amount that it was undetectable by the equipment used. Labeled sulfate Ringer's solution was in contact with the frog skin for only 1.5 hours, whereas the experiments presented previously lasted for 2 hours. However, it is believed that 1.5 hours was sufficient, and that the results obtained add further evidence for the very low sulfate permeability of frog skin. It should be said that in order to obtain a noticeable increase in count rate, which

would indicate that labeled sulfate had passed through the frog skin, an increase of only 30 counts per minute above background was needed. This increase did not occur.

D. THE INFLUENCE OF pH ON THE POTENTIOMETRIC RESPONSE
OF THE EPIDERMIS TO $[Na^+]_o$

Twelve experiments were conducted, and the results are shown in Figure 12 which is a diagram of P.D. in mV versus pH. It clearly can be seen that when the $[H^+]_o$ of the solution at the epidermis was increased, the skin potential was decreased. However, a thousand-fold change in the H^+ concentration caused only a slight decrease in P.D.

E. POTENTIOMETRIC STUDIES ON THE EPIDERMIS OF FROG SKIN
OF ANIMALS PRETREATED WITH EPINEPHRINE

The results of seven experiments are shown in Table 6. Two kinds of observations were made:

1) There was a sharp reduction of the total skin P.D. Normal skins in sulfate Ringer's can show a P.D. of nearly 100 mV, and occasionally higher values (see Table 3). Since the total skin P.D. is the sum of the P.D.'s generated at the "outer border" and the "inner border", it is conceivable that the low skin P.D. seen in the skins of epinephrine treated frogs is the result of the action of epinephrine on either or both borders. No experiments were conducted at this time to further analyze this observation.

2) It can be seen that the epidermis has almost completely lost its ability to respond to changes in $[Na^+]_o$.

DISCUSSION

A. HYPOTHESIS OF DIFFUSION DELAY

As mentioned in the Introduction, there is little reason to doubt that Na^+ ions are the chief ions, and probably the only ions, involved in the generation of the P.D. across the "outer border" of the frog skin epidermis. The "outer border" does indeed behave like a sodium-specific selective membrane. From the data presented, it is also evident that a ten fold change in $[\text{Na}^+]_o$ does not give a P.D. change of 58 mV as one would expect if the "outer border" were sodium selective. The response deviates greatly from the expectation. The lowest response was a change of 17 mV, and the highest, 35 mV for a ten fold change in $[\text{Na}^+]_o$. Hence, using the highest response, the actual (in contrast to the theoretical) response of the "outer border" is given by:

$$V_{ob} = 35 \log \frac{[\text{Na}^+]_o}{[\text{Na}^+]_c} \quad (7)$$

To bring these facts into harmony, and in an attempt to derive an equation for the "outer border" skin P.D. which is in agreement with the actual measurement, the hypothesis was made that the form of Equation (3) applies to V_{ob} . The theoretical values for V_{ob} may be obtained if one replaces in Equation (3), $[\text{Na}^+]_o$ with C_x (the Na^+ concentration in the immediate vicinity of the "outer border"). This concentration conceivably could be considerably below $[\text{Na}^+]_o$ because of diffusion delay in the regions in front of the

sodium selective "outer border." The modified equation can be expressed as:

$$V_{ob} = 58 \log \frac{C_x}{[Na^+]_c} \quad (8)$$

Figure 13 illustrates the approach to finding an answer to the problems stated above. Line A in this figure is the plot of Equation (3) showing point M, where $V_{ob} = 58$, $[Na^+]_o = 100$, and $[Na^+]_c = 10 \mu\text{Eq/ml}$. Line B is a representation of one experimental result in which $V_{ob} = 35 \text{ mV}$ for a ten fold change in $[Na^+]_o$. From an inspection of Figure 13, it is suggested that the value for V_{ob} which is lower than expected could result from the fact that the effective Na^+ concentration near the "outer border," C_x , is only $40.2 \mu\text{Eq/ml}$ when the bulk Na^+ concentration in the solution, $[Na^+]_o$, is $100 \mu\text{Eq/ml}$. Now the hypothesis is made that a Na^+ concentration gradient is established between the solution at the epidermal side of the skin and the solution in the vicinity of the "outer border." Such a gradient could develop if the rate of Na^+ diffusion from the solution to the "outer border" were slow, relative to the rate of net active Na^+ transport across the whole skin.

To derive a new relationship linking V_{ob} to $[Na^+]_o$, $[Na^+]_c$, ϕ , the rate of net active Na^+ transport, and the Na^+ permeability coefficients of the layers in front of the "outer border," it is proposed that a skin model as shown in Figure 14 may be reasonably adequate. The net Na^+ flux

across the "outer border" is given by:

$$\phi = \frac{[Na^+]_o - [Na^+]_c}{\frac{1}{P_L} + \frac{1}{P_D} + \frac{1}{P_{ob}}} \quad (9)$$

This relationship is easily obtained from Equation 64 given by Jacobs (1935). In Equation (9), P_L , P_D , and P_{ob} are the Na^+ permeability coefficients of the unstirred fluid layer (Dainty and House, 1966), the portion of the epidermis in front of the stratum germinativum, and the "outer border," respectively.

The permeability properties of the "inner border" where the active Na^+ transport mechanism involving $Na^+ \rightleftharpoons K^+$ exchange presumably occurs (Huf, 1955; Koefoed-Johnsen, 1958) are not of concern here. However, it is important to state that the assumption is made that net Na^+ transport across the whole skin is not limited by the rate of the active Na^+ transport mechanism. P_L must be on the order of 1×10^{-3} cm/sec. This is calculated from $P_L = D_L/d_L$ where D_L is the Na^+ diffusion coefficient and d_L is the thickness of the unstirred fluid layer. Inserting the values proposed by Dainty and House (1966) and by Kidder et al., (1964), one obtains $P_L = 4.0 \times 10^{-6}$ cm²/sec divided by 40×10^{-4} cm = 1×10^{-3} cm/sec. If the "outer border" is identical with the outer aspect of the cell membranes of the stratum germinativum (as it well might be), it becomes also evident that $P_{ob} \gg P_D$. The thickness of cell

membranes is on the order of 100 \AA ($1 \times 10^{-6} \text{ cm}$). Thus, even if movement in the cell membrane ("outer border") is slowed down by a factor of 10,000, applied to the diffusion coefficient of freely diffusing Na^+ ($1 \times 10^{-5} \text{ cm}^2/\text{sec}$), P_{Ob} would be relatively large, namely $1 \times 10^{-3} \text{ cm/sec}$. Thus, both P_{L} and P_{Ob} are very likely several hundred times larger than P_{D} , for which Winn et al. (1964) have figured a value on the order of $1 \times 10^{-6} \text{ cm/sec}$. This suggests that the essential diffusion barrier in the epidermis is the layer D, the intrinsic layer of Dainty and House (1966), which will be referred to here as the Dainty-House layer. Accordingly, one can write for the net Na^+ flux, ϕ , across the Dainty-House layer:

$$\phi = \frac{[\text{Na}^+]_o - C_x}{1/P_D} \quad (10)$$

Solving Equation (10) for P_D gives

$$P_D = \frac{\phi}{[\text{Na}^+]_o - C_x} \quad (11)$$

Thus, values for P_D may be obtained for varying $[\text{Na}^+]_o$ if ϕ and C_x are known. This will be discussed under 1) and 2) below.

The skin model suggested in Figure 14 implies that in the steady state, the K^+ concentration near the "outer border" (in the C_x region) is elevated above the K^+ concentration in the bath. The model also indicates how this local

increase in $[K^+]$ is obtained. K^+ ions of the bath move across the K^+ selective "inner border" and enter the C_x region via the extracellular space, since they cannot pass through the Na^+ selective "outer border." Therefore, in the steady state (Fig. 15), $C_x \ll [Na^+]_o$, $[SO_4^{2-}]_x = [SO_4^{2-}]_o$ (since sulfate ions are not removed during Na^+ transport from the "outer border"), and $[K^+]_x \gg [K^+]_o$. In this manner, electroneutrality is preserved. The question arises if it can be shown that skins in sulfate Ringer's have a higher total K^+ content than control skins kept in chloride Ringer's. A simple calculation, based on the data in Figure 15 shows that the maximal increase in skin K^+ that must occur if the model is correct is 6% of the total skin K , which is $1.2 \mu\text{Eq}/\text{cm}^2$. This is calculated as: $(70-10) \mu\text{Eq}/\text{cm}^3 \times 12 \times 10^{-4} \text{ cm}^3 = 0.072 \mu\text{Eq } K^+$. The 6% increase in skin K^+ is certainly an over-estimation, since it was assumed that the total epidermal region in front of the "outer border" had a value of $[K^+] = 70 \mu\text{Eq}/\text{ml}$. It is more appropriate, however, that only the region in the immediate vicinity of the "outer border" (a region of only 2-3 μ thick), need be at this high K^+ concentration. It is obvious from this discussion that an increase of only a few % in the increase in total skin K^+ , could not readily be observable by ordinary flame photometric methods. Friedman and Huf (unpublished data) did not find a difference in the K^+ content of skins kept in sulfate and chloride Ringer's.

1) DATA ON Na^+ FLUX (ϕ)

Data are available in the literature concerning the dependence of net Na^+ flux on the Na^+ concentration at the epidermal side of the skin, $[\text{Na}^+]_o$. The information most applicable to this paper is that published in 1949 by Ussing. Changing $[\text{Na}^+]_o$ from 2 to 170 mM/l, this author found that both Na^+ outflux (inside \rightarrow outside of the skin), and especially Na^+ influx (outside \rightarrow inside of the skin) increased with increasing $[\text{Na}^+]_o$. Hence, net Na^+ flux (influx minus outflux) also increased with increased $[\text{Na}^+]_o$. When the "open skin system," i.e., the skin not short-circuited, was used, maximal net sodium flux was found to be on the order of $1 \mu\text{M} \times \text{cm}^{-2} \times \text{hr}^{-1}$, when the anion was chloride supplied by chloride Ringer's.

In view of the fact that so many skin parameters have been measured in sulfate Ringer's, it is somewhat surprising that net Na^+ flux data on skins in sulfate Ringer's have not been published. Unpublished work from Dr. E. G. Huf's laboratory shows that for skins in 55 mM Na^+ /l Na_2SO_4 solution, the net sodium flux is 50% of the net sodium flux of skins in chloride Ringer's. Time did not permit measurement of the dependence of sodium flux on $[\text{Na}^+]_o$ when the skin is bathed in sulfate Ringer's. If the reasonable assumption is made that the relationship is similar to the one that Ussing has shown to exist for skins in chloride Ringer's, data concerning ϕ can be obtained from the work of Ussing and the investigations in Dr. Huf's laboratory, as noted above. Na^+ flux values obtained in the manner are

given in Table 7, column 2. In Figure 16, the reciprocal of the flux, $1/\phi$, is plotted against the reciprocal of $[\text{Na}^+]_o$. The equation for the regression line is:

$$\frac{1}{\phi \times 10^4} = 0.72 + 38 \frac{1}{[\text{Na}^+]_o} \quad (12)$$

2) DATA ON C_x

When the ideal response of the "outer border" (58 mV) is compared with the actual response (35 mV), it can be seen from Figure 13 (comparing lines A and B), that the skin behaves as if the sodium concentration at the "outer border," C_x , is only a fraction of the sodium concentration in the bathing solution, $[\text{Na}^+]_o$. For instance, C_x is apparently 40.2 $\mu\text{Eq Na/ml}$, whereas the $[\text{Na}^+]_o$ is 100 $\mu\text{Eq Na/ml}$. Making use of Equations (7) and (8), the values of C_x that satisfy Equation (8) can be calculated from the potentiometric response curve B. To carry out these calculations requires knowledge of $[\text{Na}^+]_c$, the intracellular Na^+ concentration. This has been estimated by Andersen and Zerahn (1963), who give a value near 10 $\mu\text{Eq Na/ml}$. $[\text{Na}^+]_c$ seems to increase somewhat with increasing $[\text{Na}^+]_o$. Insufficient data are available at present, and therefore this complicating factor has been neglected in the following calculations, which will have to be refined later. The calculations of C_x are facilitated by combining Equations (7) and (8) and solving for C_x . One obtains:

$$C_x = [\text{Na}^+]_c^{(1-k)} \times [\text{Na}^+]_o^k \quad (13)$$

in which $k = 35/58 = 0.604$. Several values of C_x were obtained in this manner for a number of arbitrarily selected values of $[\text{Na}^+]_o$. The results are given in Table 7, column 3.

B. CALCULATION OF THE Na^+ PERMEABILITY COEFFICIENT IN THE DAINTY-HOUSE LAYER

For the calculation of the permeability coefficient of Na^+ in the Dainty-House layer Equation 11 was applied. Values for P_D are given in Table 7, column 4. When a plot is made of the reciprocal of P_D against $[\text{Na}^+]_o$, an approximately linear relationship results (Fig. 17). The straight line which has been drawn is somewhat arbitrary. Because of the obvious deviations from the linear relationship at low values for $[\text{Na}^+]_o$, it was assumed that the point of intersection on the ordinate was at 0.05×10^{-5} . The justification for this is that by using this approximation, one is able to derive an equation for the electrical response of the epidermis to varying $[\text{Na}^+]_o$. As shown below, this explains in a logical manner the failure of the response to follow the ideal Nernst law. The equation describing the relationship between P_D and $[\text{Na}^+]_o$ (Fig. 17) is:

$$\frac{1}{P_D \times 10^5} = 0.05 + 0.067 [\text{Na}^+]_o \quad (14)$$

A similar empirical relationship was obtained by Cereijido et al., (1964). These authors used a three-compartment skin model and analyzed the movement of Na^{24} from the epidermis to the corium of the skin. The rate of movement of Na^{24} across the "outer border" could be characterized by a rate constant k_{12} . This is related to the permeability coefficient of Na^+ across the "outer border" in the following way: $P_{\text{Na}}^{\text{O}} = 1.59 \times k_{12}$; the factor 1.59 is explained in the paper cited. The dependence of P_{Na}^{O} on $[\text{Na}^+]_{\text{O}}$ could be expressed as:

$$\frac{1}{P_{\text{Na}}^{\text{O}} \times 10^5} = 0.08 + 0.013 [\text{Na}^+]_{\text{O}} \quad (15)$$

For comparison of data this line (B) is also shown in Figure 17. The smaller slope of line (B) as compared to (A) means that the P_{Na}^{O} values as given by Cereijido et al. are greater than the P_{D} values of the present work. It must be pointed out, however, that Cereijido et al. performed their measurements on short circuited skins in chloride Ringer's, in which NaCl was replaced by choline chloride when lower $[\text{Na}^+]_{\text{O}}$ was needed. In contrast, the measurements described in this thesis were made on open skins in solutions containing Na_2SO_4 . It will be noted from the data in Table 7 that the sodium flux values, ϕ , are several times greater in the former than in the latter case. The increase in the Na^+ permeability coefficient with decreasing $[\text{Na}^+]_{\text{O}}$.

is expressed by the similar Equations (14) and (15).

Although no explanation for these relationships can be given, the great similarity of results strengthens the hypothesis of diffusion delay and active Na^+ transport across the skin as the chief factors which explain the failure of the Nernst law when testing the electrical response of the epidermis to changing $[\text{Na}^+]_o$.

C. EFFECT OF pH ON P.D.

If an increase in skin P.D. with increasing $[\text{H}^+]_o$ had occurred, it might be suggested that this was the result of H^+ moving across the "outer border" as predicted by the Hodgkin-Katz equation. Since the observations regarding the effect of pH are contrary to the expectation if the Hodgkin-Katz equation had been applicable, it is concluded that the permeability of the "outer border" for H^+ is not significant in the generation of the fraction of the total skin P.D. that has its origin at the "outer border." In weighing the importance of the results shown in Figure 12, it must also be kept in mind that here a thousand fold change in $[\text{H}^+]_o$ was investigated. The reason why a decrease in pH within reasonable limits (pH 9 to pH 6) resulted in a slight to moderate decrease in skin P.D. can not be given. With regard to the hypothesis that has been presented, it is suggested that an increase in $[\text{H}^+]_o$ leads to a decrease of the Na^+ permeability coefficient in the Dainty-House layer. This would lead to a lowering of the Na^+ concentration near

the "outer border," C_x , and thereby reduce the Na^+ concentration gradient across the "outer border"; this would lead to a lowering of the skin P.D. as shown by the present data.

D. EFFECT OF EPINEPHRINE ON SKIN P.D.

Since it is known from the literature (Cereijido and Curran, 1965) that about half of the total skin P.D. is generated at the "outer border," and the other half at the "inner border," it seems to follow that epinephrine must, in part, have acted on the "outer border," the properties of which are the main concern of this thesis.

For skins under the influence of epinephrine, it must be assumed that the Na^+ concentration near the "outer border," C_x , was high, probably as high as the Na^+ concentration in the solution $[\text{Na}^+]_o$. Epinephrine is known for its property of increasing membrane permeability. With C_x being high, rather than low as in the control skins, the low P.D. at the "outer border" and its lack of response to changes in $[\text{Na}^+]_o$ can only mean that epinephrine drastically changed the ion selectivity of the "outer border." It is concluded that under the influence of epinephrine, the "outer border" loses its specific Na^+ selectivity, and thus, did not act as a source for generation of a Nernst type diffusion potential.

Epinephrine is known to stimulate the secretion of mucus (Watlinton et al., 1965) from the skin glands (which

are seen in the photomicrograph shown in Figure 2). The question arises whether the low total skin P.D. and the relative insensitivity of the epidermis to changes in $[Na^+]_o$ is explainable on the basis of an altered function of epinephrine stimulated skin glands. It is unlikely that this is the case. Following epinephrine injection, the mucus that pours over the surface of the skin is alkaline (pH 7.5). This in itself should lead to an increase, and not to a decrease, in skin P.D.

It is also known that under the influence of epinephrine, there occurs an active outward transport of Cl^- ions (Koefoed-Johnsen et al., 1952) and possibly of $SO_4^{=}$ ions (Campbell et al., in print). With everything else remaining constant, this also should lead to an increase in skin P.D. (outside more negative relative to the inside). Therefore, the conclusion is that epinephrine destroys the physiochemical properties of the "outer border" in such a way that it loses its normally high Na^+ selectivity.

E. EQUATION FOR THE "OUTER BORDER FROG SKIN P.D.

On the basis of the hypothesis of diffusion delay of Na^+ in the outer regions of the epidermis of frog skin (the Dainty-House layer), an equation that describes adequately the electrical response of the frog skin epidermis to varying $[Na^+]_o$ can be derived from Equation (11):

$$C_x = \frac{[Na^+]_o P_D - \phi}{P_D} \quad (16)$$

When this expression for C_x is inserted into Equation (8):

$$V_{ob} = 58 \log \frac{[Na^+]_o P_D - \phi}{[Na^+]_c P_D} \quad (17)$$

Upon changing from a solution I to a solution II with Na^+ concentrations of $[Na^+]_o^I$ and $[Na^+]_o^{II}$

$$\Delta V_{ob} = 58 \log \frac{[Na^+]_o^{II} - (\phi/P_D)^{II}}{[Na^+]_o^I - (\phi/P_D)^I} \quad (18)$$

It will be recognized that the term ϕ/P_D has the dimension of "concentration." This equation shows that the modified Nernst equation does hold if either ϕ is zero or P_D is great. In other words, if little Na^+ is transported away from the "outer border" and, or, if the Na^+ permeability coefficient of the layers in front of this border is great, then the effective Na^+ concentration at the "outer border" (C_x) is the same as the Na^+ concentration in the solution $[Na^+]_o$. If this is the case, then for $[Na^+]_o^{II} = 0.1 [Na^+]_o^{II}$, ΔV_{ob} will be -58 mV.

However, a secondary complication may be visualized. If Na^+ flux suddenly should become zero, then a short time later, the Na^+ concentration gradient across the "outer border" might disappear because of increase in the intracellular Na^+ concentration, and the source of a P.D. at this border might then disappear. This secondary complication, however, does not invalidate the usefulness of

Equation (18). Ordinarily, net flux does take place, and P_D is relatively small. Therefore, ΔV_{Ob} is expected to be less than -58 mV when changing $[Na^+]_o$ from 100 to 10 mM/l.

It has been pointed out that both ϕ and P_D are dependent on $[Na^+]_o$. The most desirable approach to simplifying Equation (18) would be to enter the relationships between ϕ , P_D , and $[Na^+]_o$ into this equation. Unfortunately, there exists at present no theory that would explain the empirical functions (Equations 12 and 14) relating these variables. It is possible, however, to give approximate empirical equations that describe the dependence of ϕ on $[Na^+]_o$, and also of P_D on $[Na^+]_o$. The equations are given above as (12) and (14). By combining these two equations, the following expression for ϕ/P_D results:

$$\phi/P_D = \frac{10 A [Na^+]_o + 10 B [Na^+]_o^2}{a [Na^+]_o + b} \quad (19)$$

$$A = 0.05; B = 0.067; a = 0.72; b = 38$$

A plot of ϕ/P_D against $[Na^+]_o$ is shown in Figure (18). The data necessary are given in Table 7, columns 1 and 5. For comparison of the present results with those obtained by Cereijido et al. (1965), ϕ/P_{Na}^o values for varying $[Na^+]_o$ were also calculated from the information given in the paper cited above. The results of the calculations are tabulated in Table 7, column 8, and a plot of ϕ/P_{Na}^o against $[Na^+]_o$ is also shown in Figure 18. One can see the same kind of dependence of ϕ/P on $[Na^+]_o$, although the experiments on

which these curves are based are quite different in nature. The differences seen in the two functions may be due to the fact that the treatment of skins was different. (p.34).

Finally, Equations (18) and (19) were used to construct a plot of ΔV_{ob} against $[Na^+]_o$ (Fig. 19). The necessary ϕ/P_D data for five arbitrarily selected $[Na^+]_o$ values are given in Table 7, column 5 (data of this thesis) and column 8 (data of Cereiido et al.). In regard to the data in this thesis, it can be seen that Equations (18) and (19) adequately describe the experimental observations and also point to the factors (ϕ and P_D) that lead to the deviation of the experimental data from the simple Nernst equation. When the ϕ/P_{Na}^o values calculated from the work of Cereiido et al. are used, the result is a response line of the "outer border" that corresponds closely to the one predicted by the unmodified Nernst equation.

Thus it appears that the Nernst equation is the limiting law that describes the electrical response of the epidermis to varying Na^+ concentrations in the salt solutions at the epidermal side of the skin. Under experimental conditions of this research, however, the electrical response is lower than expected because of the occurrence of continuous active Na^+ transport across the skin, and because of diffusion delay in the regions (Dainty-House layer) in front of the Na^+ selective border.

SUMMARY

1. Experiments were conducted to examine the effect of varying Na^+ concentrations at the epidermal "outer border" on the P.D.
2. Theoretical and experimental evidence was given in support of sodium ions being the major contributors to the frog skin potential at the "outer border."
3. It was hypothesized that active Na^+ transport across the frog skin and diffusion delay in the Dainty-House layer account for the discrepancy between the theoretical Nernst response and the actual response of the frog skin to changing $[\text{Na}^+]_o$.
4. Taking these two factors into account, a modified Nernst equation was proposed which adequately describes the P.D. response of the epidermis to varying $[\text{Na}^+]_o$.
5. S^{35} labeled sulfate experiments indicate that frog skin is impermeable to sulfate.
6. Epinephrine treated skins lost their ability to respond to varying $[\text{Na}^+]_o$.

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FIGURE 1

Cross section of the epidermis.

- A. Stratum corneum
- B. Stratum spinosum and granulosum
- C. Stratum germinativum
- D. Dermis

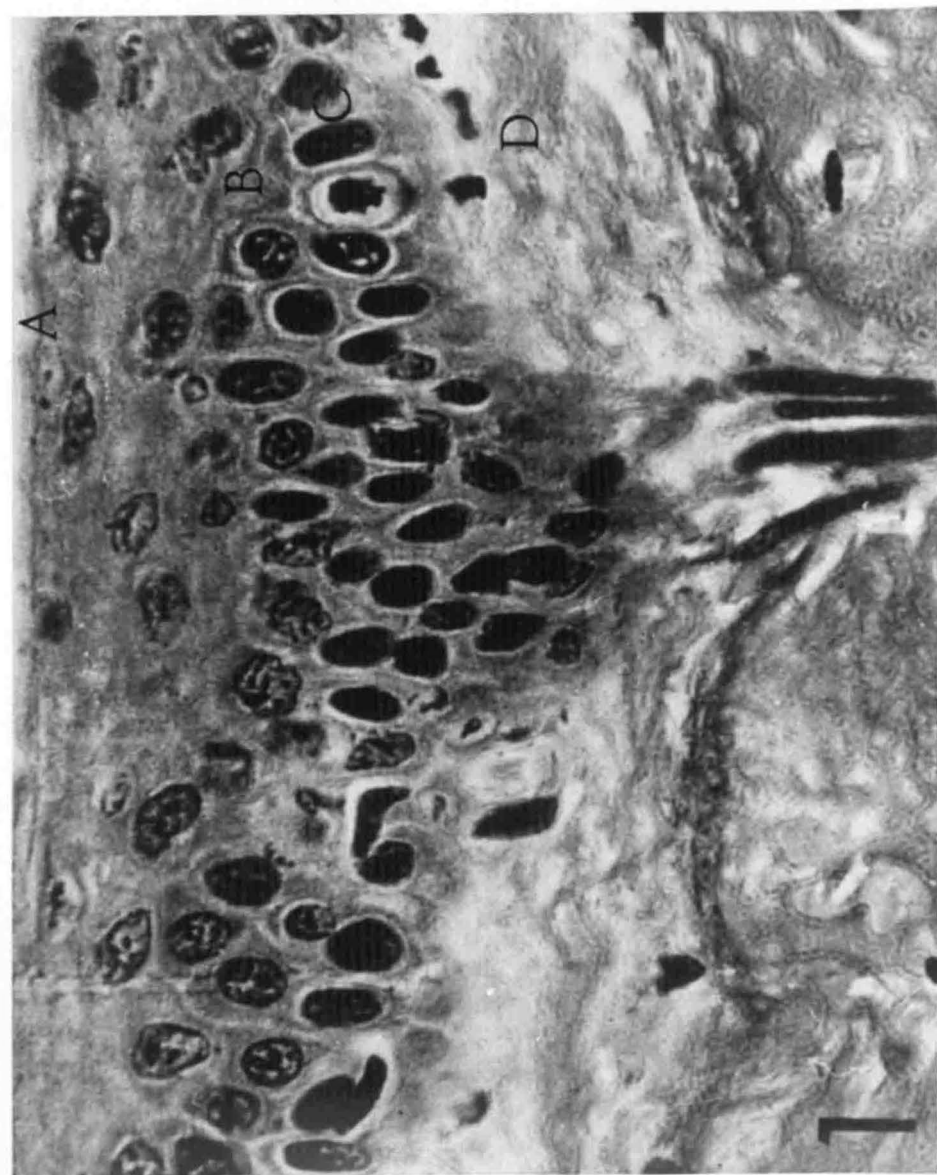


FIGURE 2

Cross section of the frog skin

A. Stratified epidermis

B. Dermis

C. Mucus gland

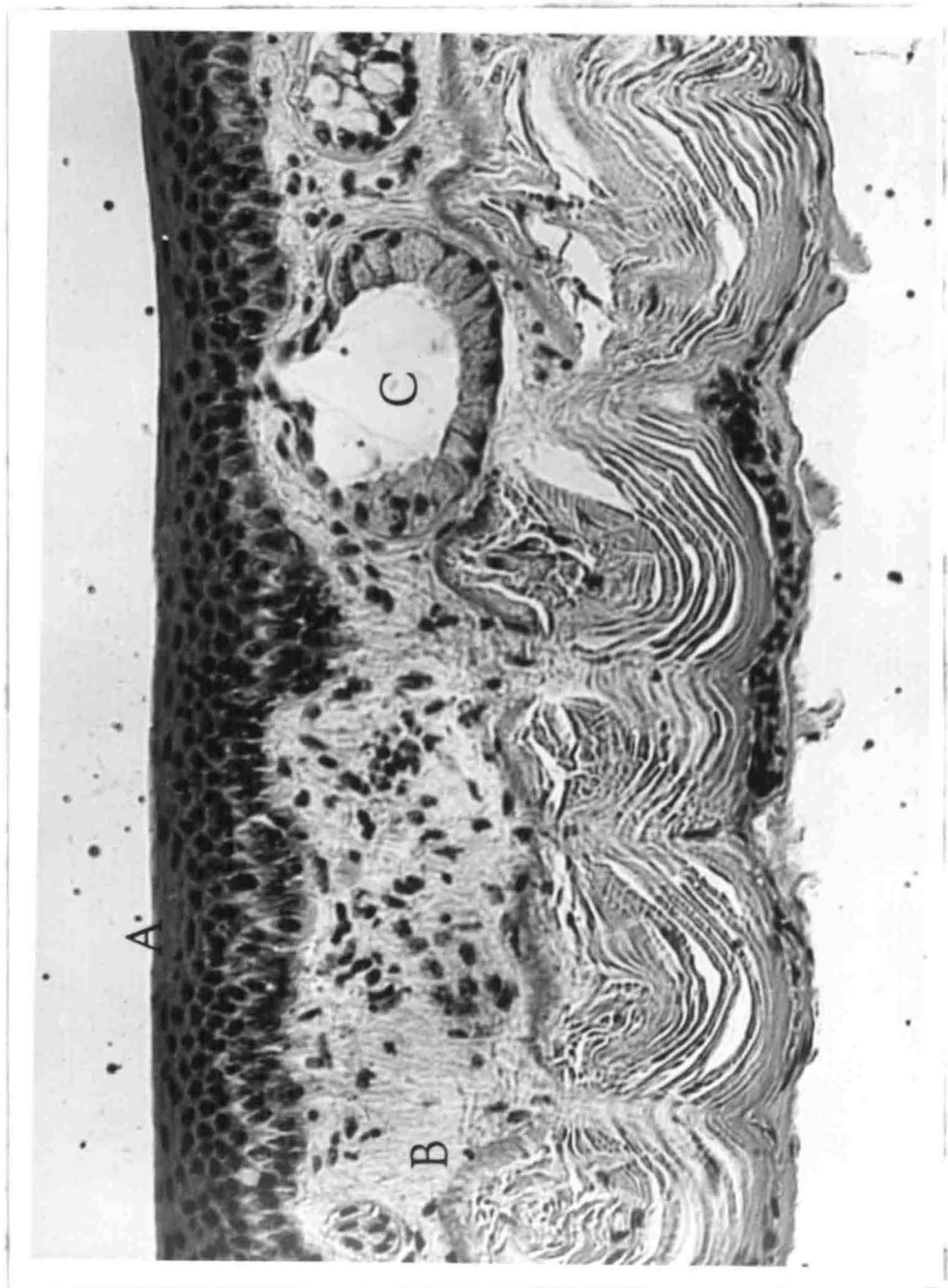


FIGURE 3

Decerebration of frog

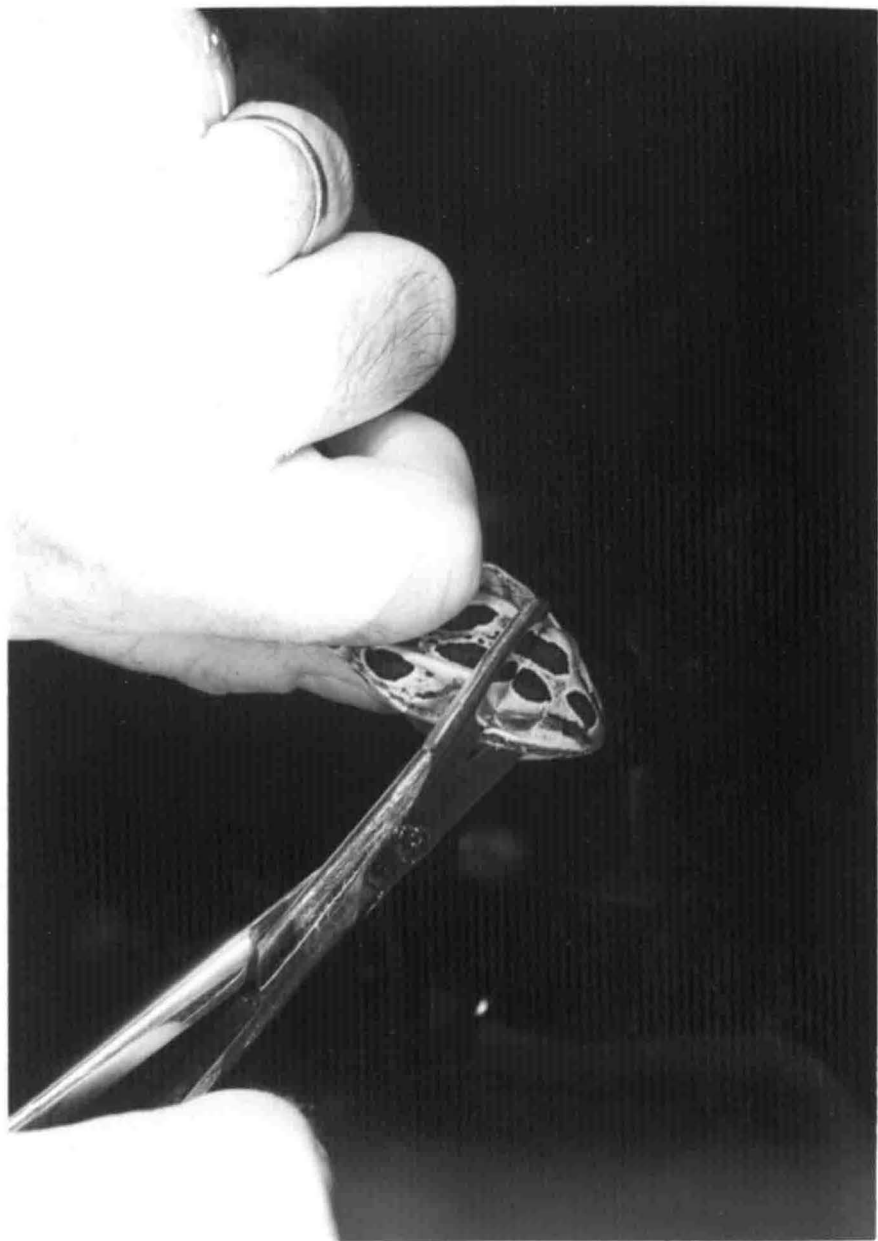


FIGURE 4

Initial incision for removing belly skin



FIGURE 5

Belly skin spread out showing shape and size

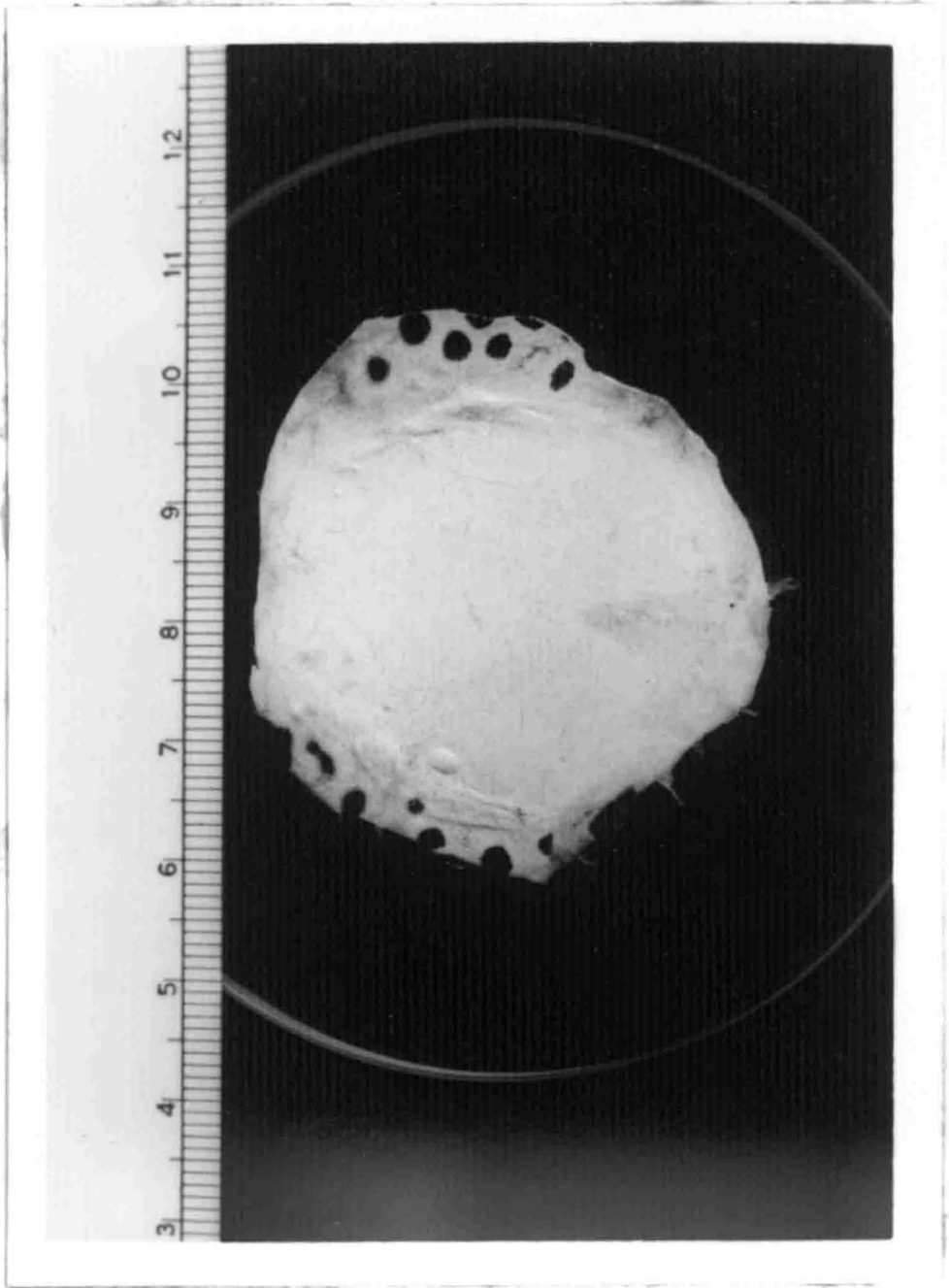


FIGURE 6

Assembling of cell

The skin is placed across the open end of one chamber; the other chamber is being placed in position.

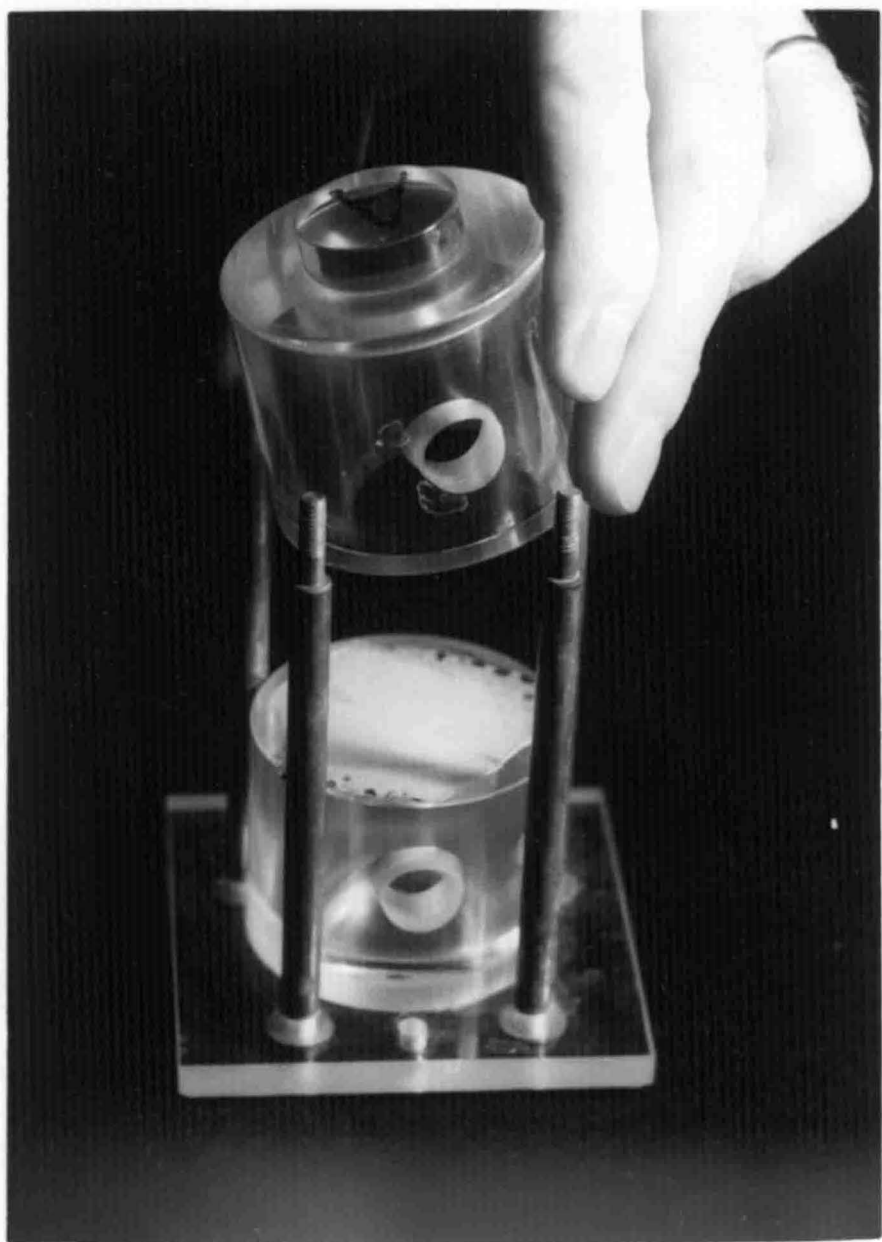


FIGURE 7

Diagram of lucite cell

(and one of the bolts to hold the chambers together)

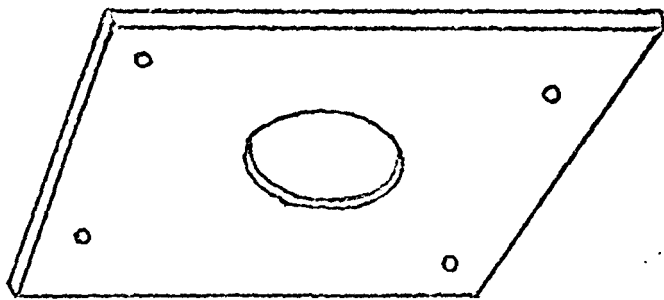
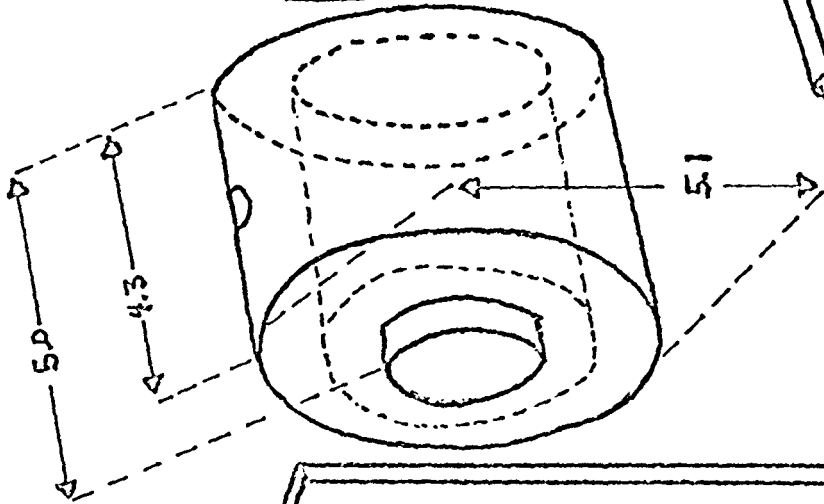
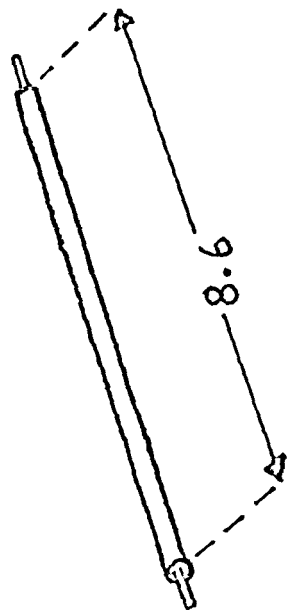
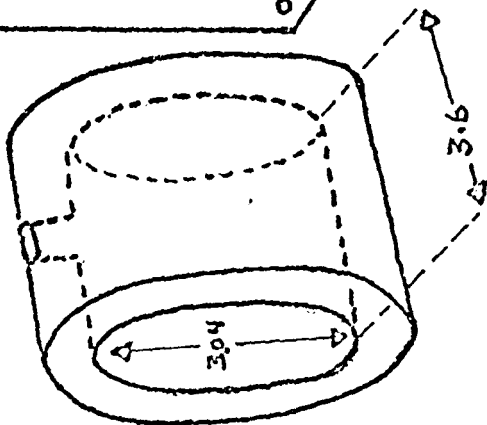
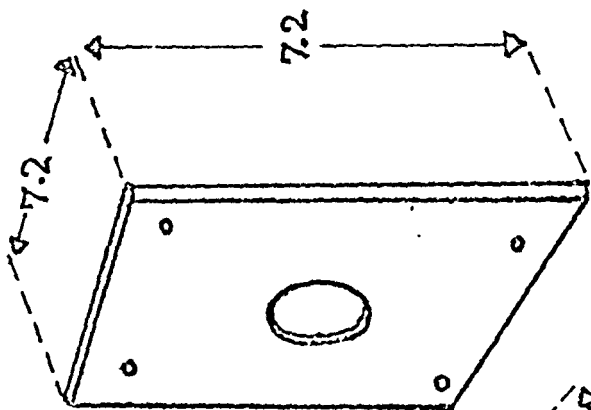


FIGURE 8

The apparatus

8A. Photograph

8B. Diagram showing:

Section #1

(a) cell positioner which held the cells in the proper position to receive the calomel electrodes.

(b) magnetic stirrers.

(c) calomel electrodes.

(d) electrode elevator.

Section #2 (Master control panel)

(e) magnetic stirrer controls.

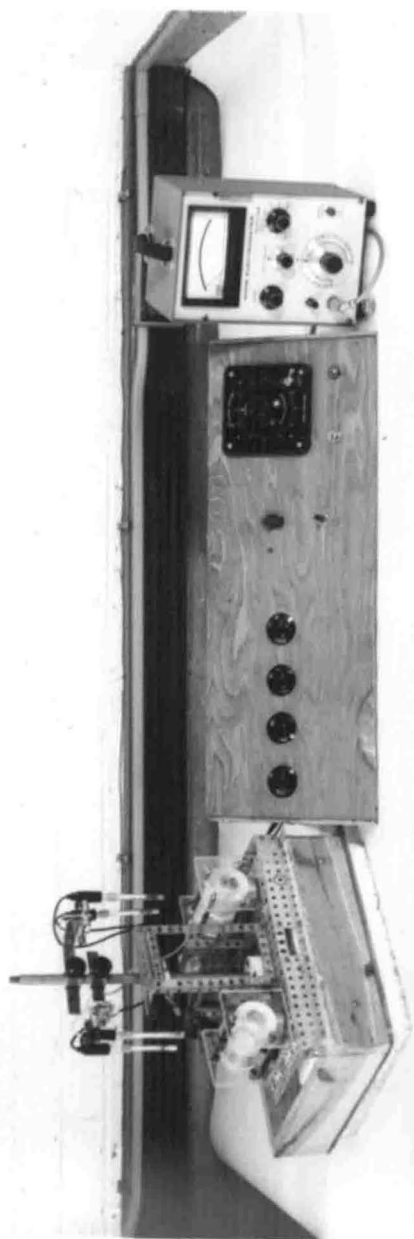
(f) electrode selector switch for selecting the electrode pair for measuring P.D.

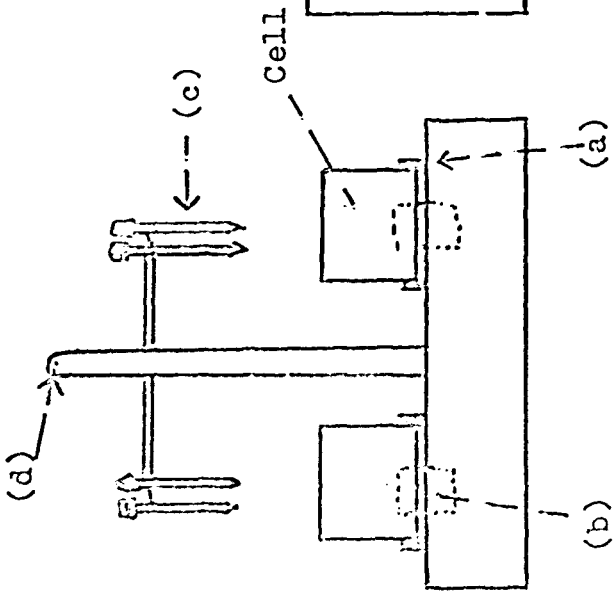
(g) electrode elevator switch for raising and lowering the electrodes.

(h) automatic timer.

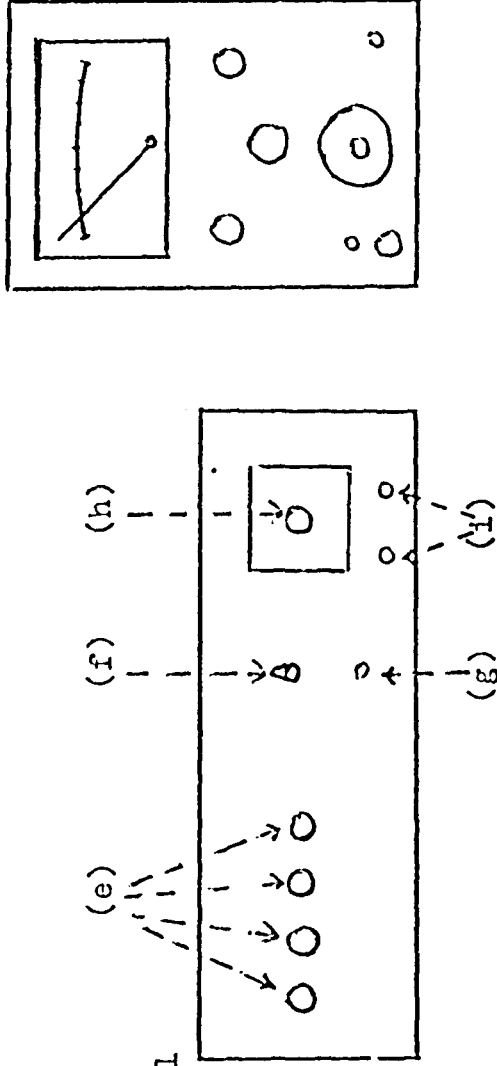
(i) timer alarm switches.

Section #3 (Keithley electrometer)

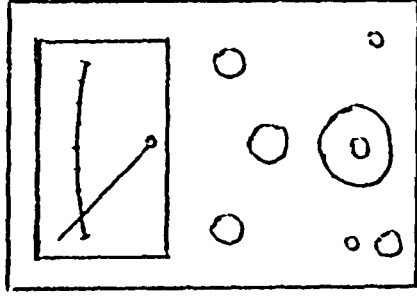




SECTION #1



SECTION #2



SECTION #3

FIGURE 9

Electrical response of the epidermis of frog skin to change in Na^+ concentration for ♂ and ♀ frogs. Theoretical Nernst response is shown as the dashed line.

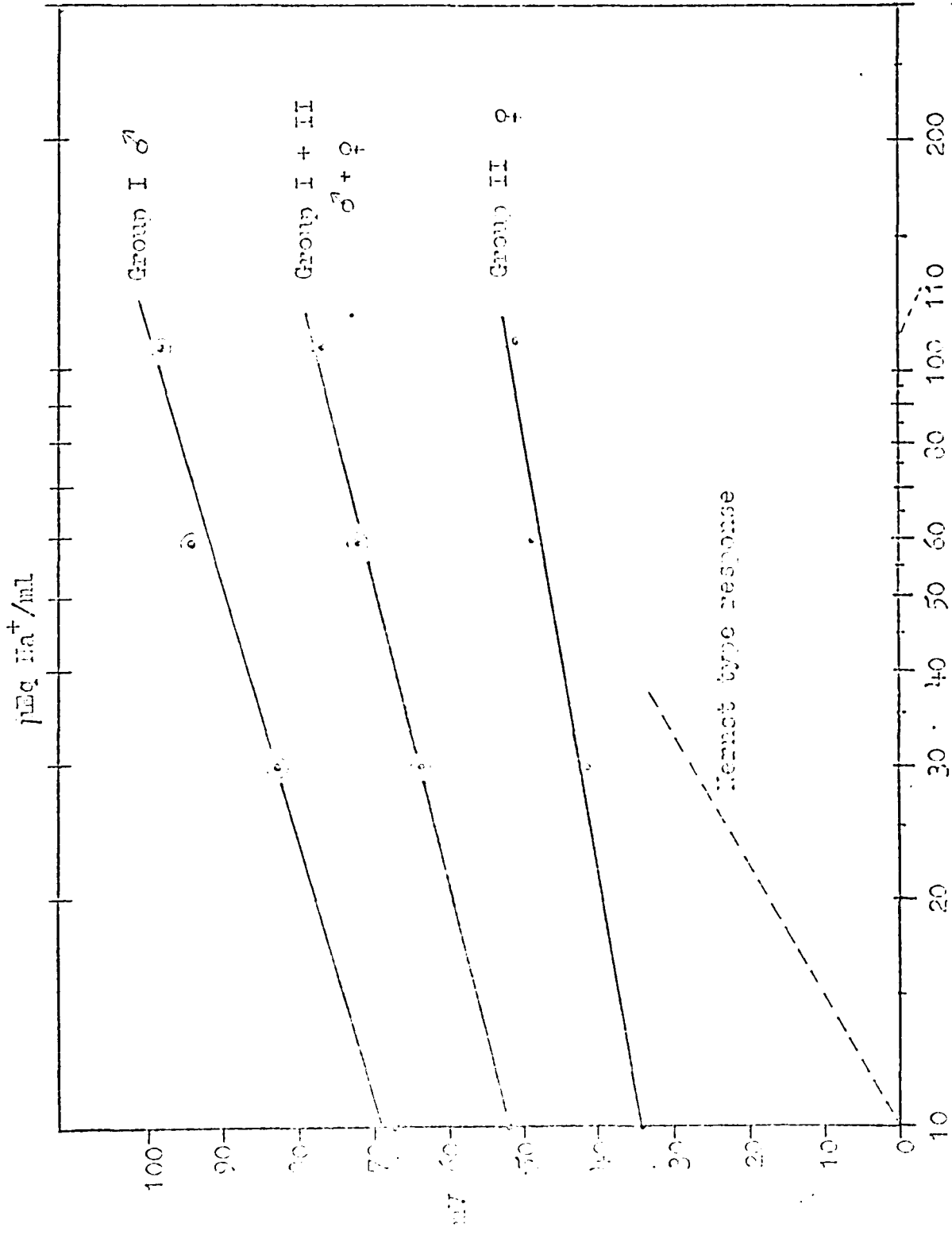


FIGURE 10

Electrical response of skins of Group III to changes in $[\text{Na}^+]_o$. The curve is broken up into two linear responses.

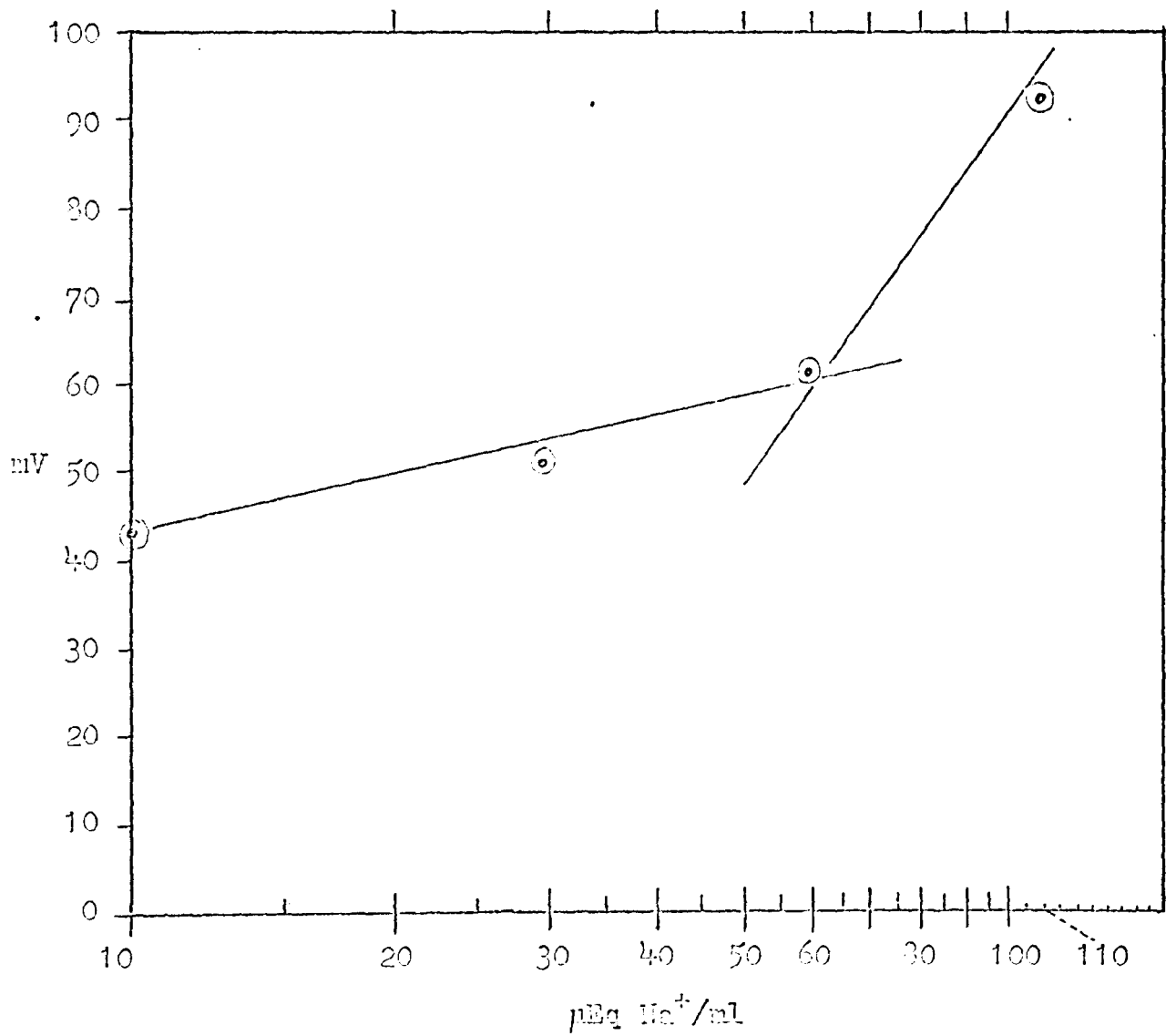
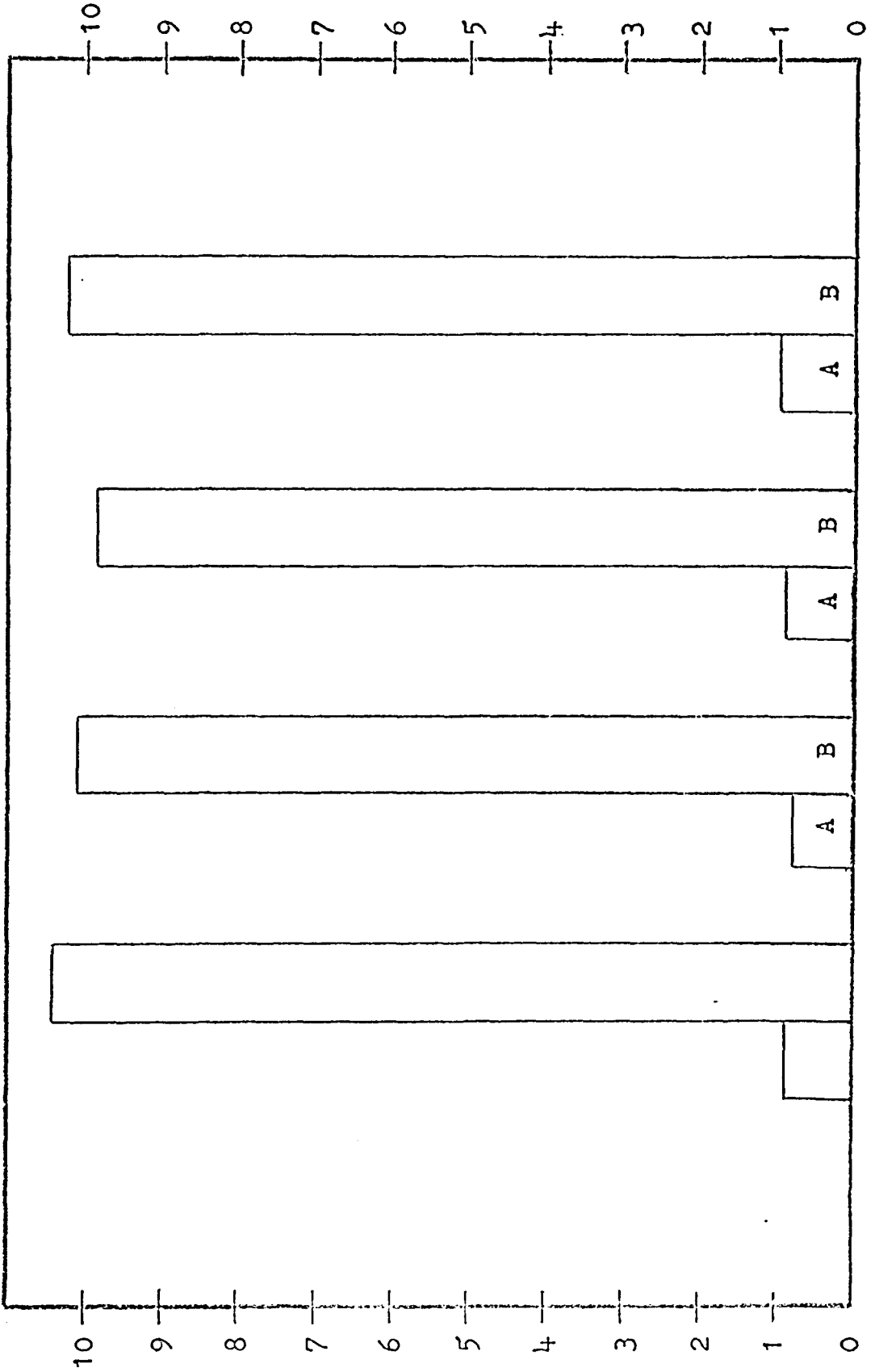


FIGURE 11

Results of $\text{SO}_4^{=}$ permeability studies using S^{35} labeled
Sulfate

Three skins (I, II, III) where B is the epidermal labeled solution and A is the dermal unlabeled solution. BG is the background in counts per minute of normal sulfate Ringer's solution and Std. is the count rate of the labeled solution. All counts were made on a 0.1 ml sample and were not corrected for background.

COUNT RATE IN THOUSANDS



COUNT RATE IN THOUSANDS/0.1 ml

BG Std. I II III

FIGURE 12

Electrical response of the epidermis to changes in pH. The skins were in sulfate Ringer's (110 mM Na/l--10 mM K/l). The graph shows a decrease in P.D. with a decrease in pH from 9 to 6. The bars represent the average P.D.'s obtained on twelve skins. The standard deviations are shown for each pH.

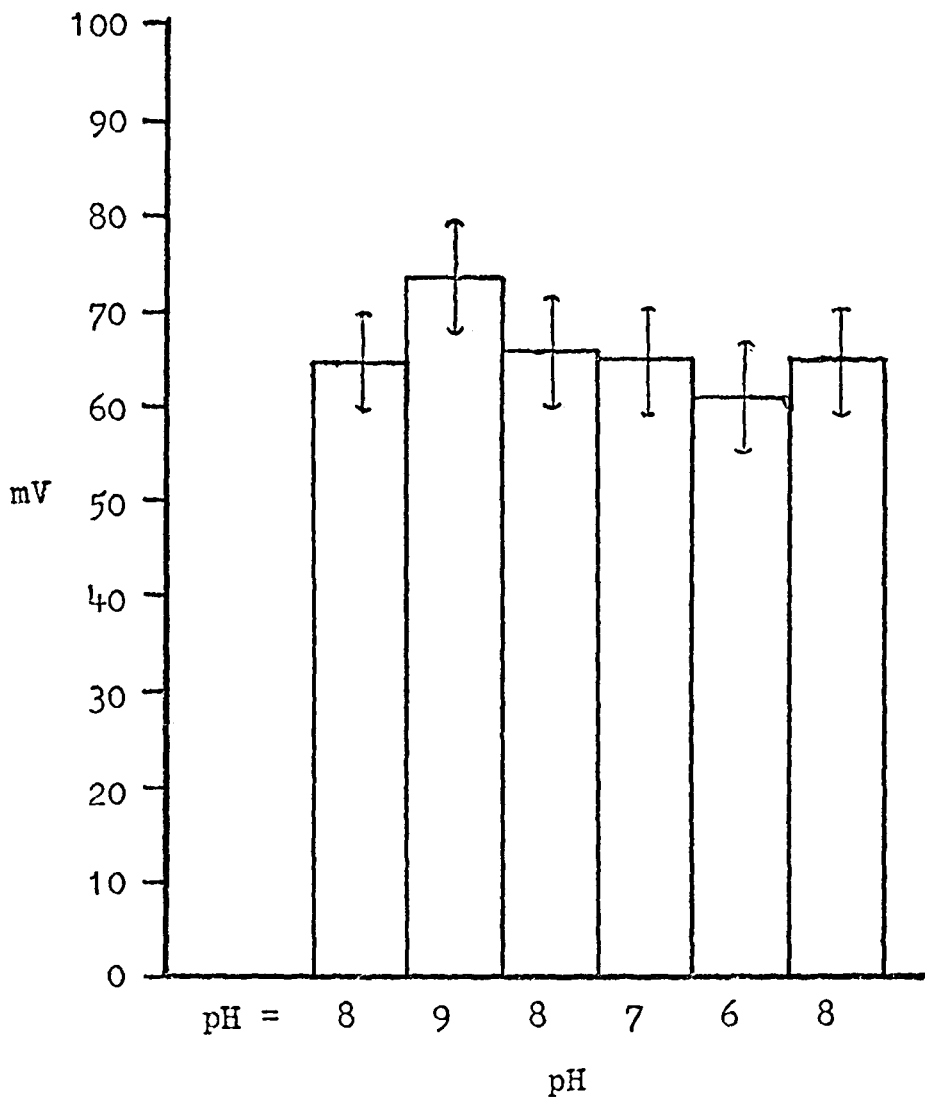


FIGURE 13

Plots of Equation (3)

Line A shows point M where $V_{ob} = 58$ for $[\text{Na}^+]_o = 100 \mu\text{Eq/ml}$ and $[\text{Na}^+]_c = 10 \mu\text{Eq/ml}$. Experimental results, line B, shows where $V_{ob} = 35 \text{ mV}$. The dashed line indicates that the Na^+ concentration at C_x is only $40.2 \mu\text{Eq/ml}$ when $[\text{Na}^+]_o = 100 \mu\text{Eq/ml}$.

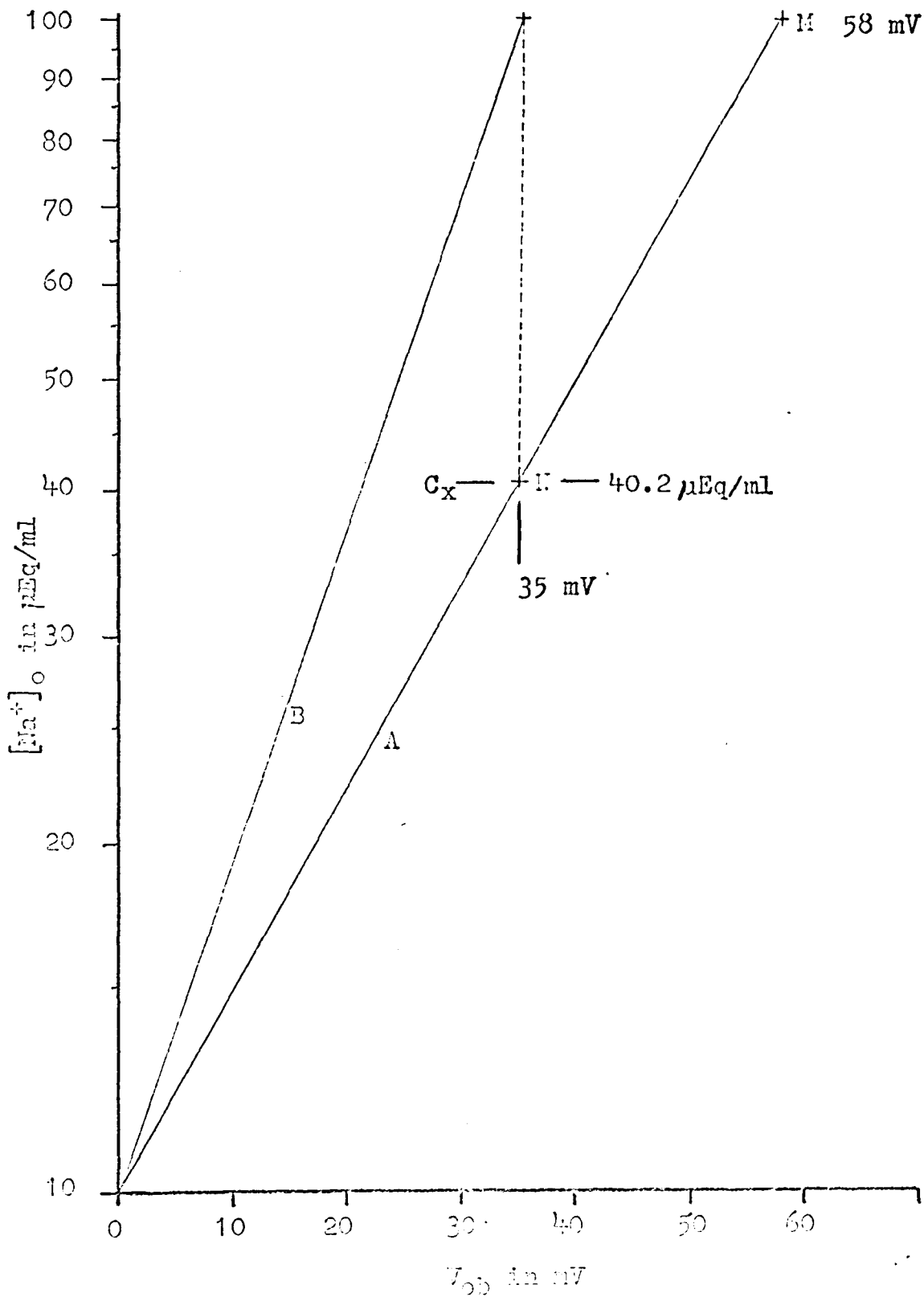


FIGURE 14

Simplified model of frog skin

The epidermis is divided into two compartments.

C = stratum corneum, stratum granulosum.

D = stratum germinativum.

ob = "outer border" assumed to be selectively permeable to sodium, impermeable to potassium and sulfate.

ib = "inner border" at which an electrogenic $\text{Na}^+ \rightleftharpoons \text{K}^+$ exchange pump operates (Winn et al., 1964).

ob and ib may be identical with the cell membranes of the stratum germinativum.

$[\text{Na}^+]_o$ = Na^+ concentration of the outside solution.

C_x = Na^+ concentration near the "outer border."

$[\text{Na}^+]_c$ = intracellular Na^+ concentration.

$[\text{Na}^+]_i$ = Na^+ concentration of the inside solution.

A^- = diffusible metabolic anions.

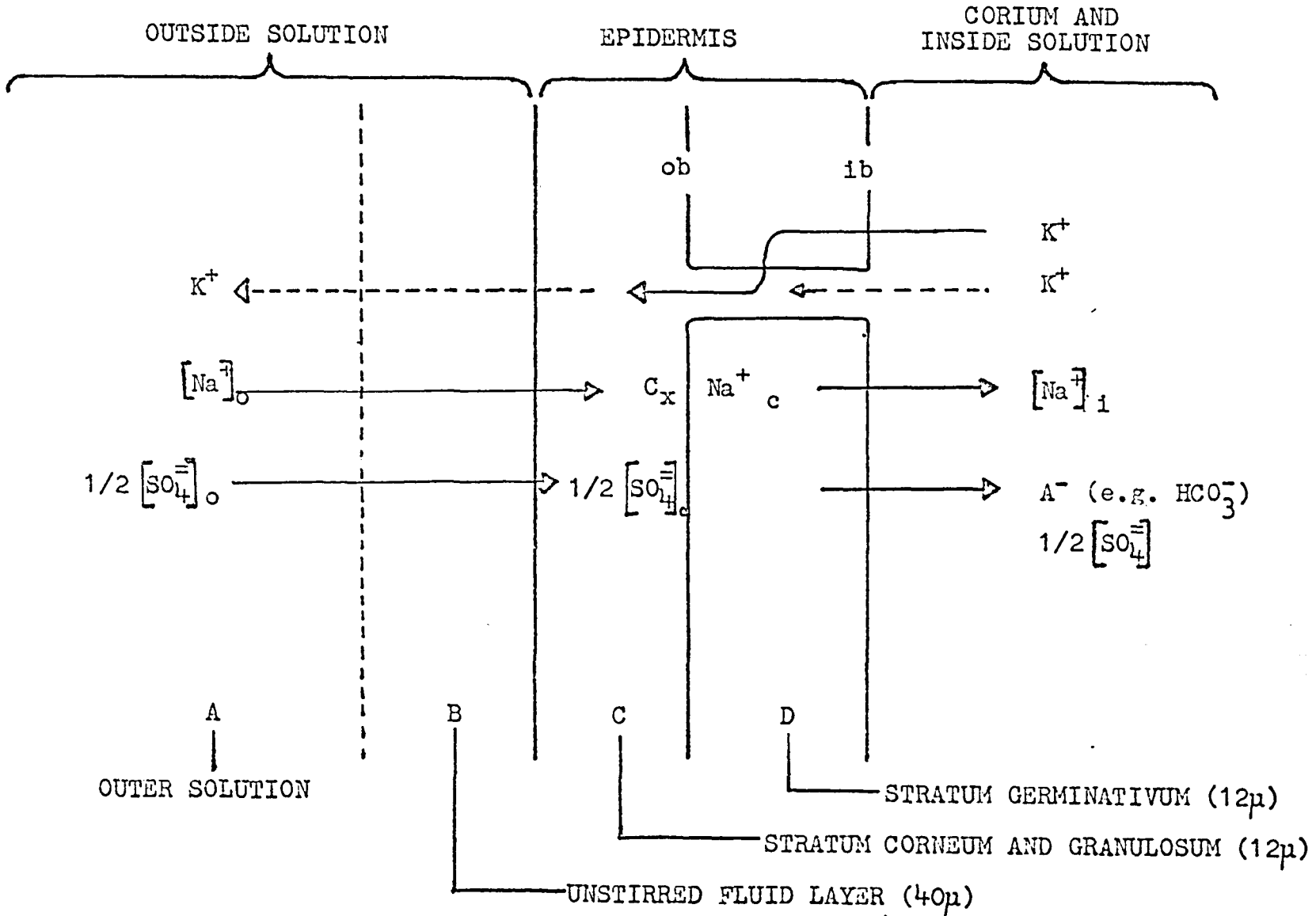


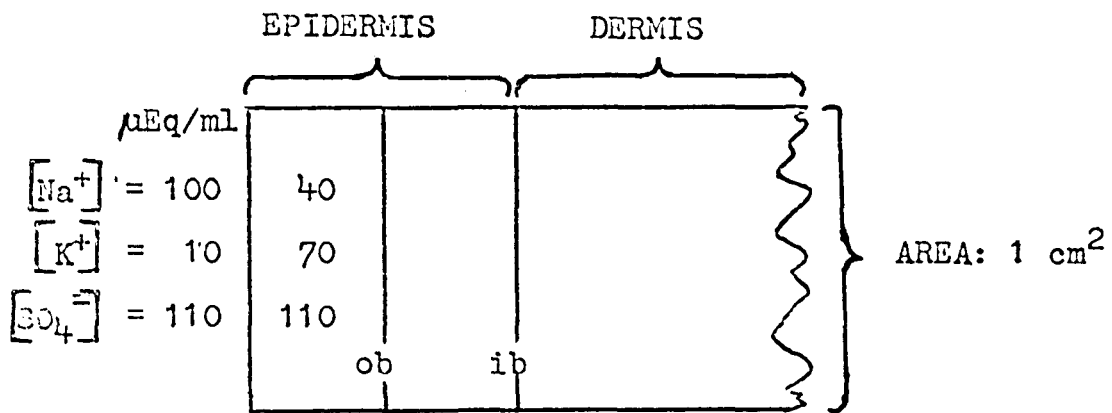
FIGURE 15

Steady state sodium and potassium distribution in frog skin

ob = "outer border"

ib = "inner border"

*Data for skin kept for 5 hours in sulfate Ringer's before chemical analysis (unpublished data of Friedman and Huf).



Thickness, μ : $\leftarrow 12 \times 12 \times 300 \rightarrow$

Volume, ml: 12×10^{-4}

Total amount of Na⁺ and K⁺ in wet skin*

Na⁺, $\mu\text{Eq}/\text{cm}^2$ $\leftarrow 2.6 \rightarrow$

-K⁺, $\mu\text{Eq}/\text{cm}^2$ $\leftarrow 1.2 \rightarrow$

FIGURE 16

Relationship between net sodium flux, ϕ , and $[\text{Na}^+]_o$.
is expressed in $\mu\text{Eq} \times \text{cm}^{-2} \times \text{hr}^{-1}$, $[\text{Na}^+]_o$ in $\mu\text{Eq}/\text{ml}$.

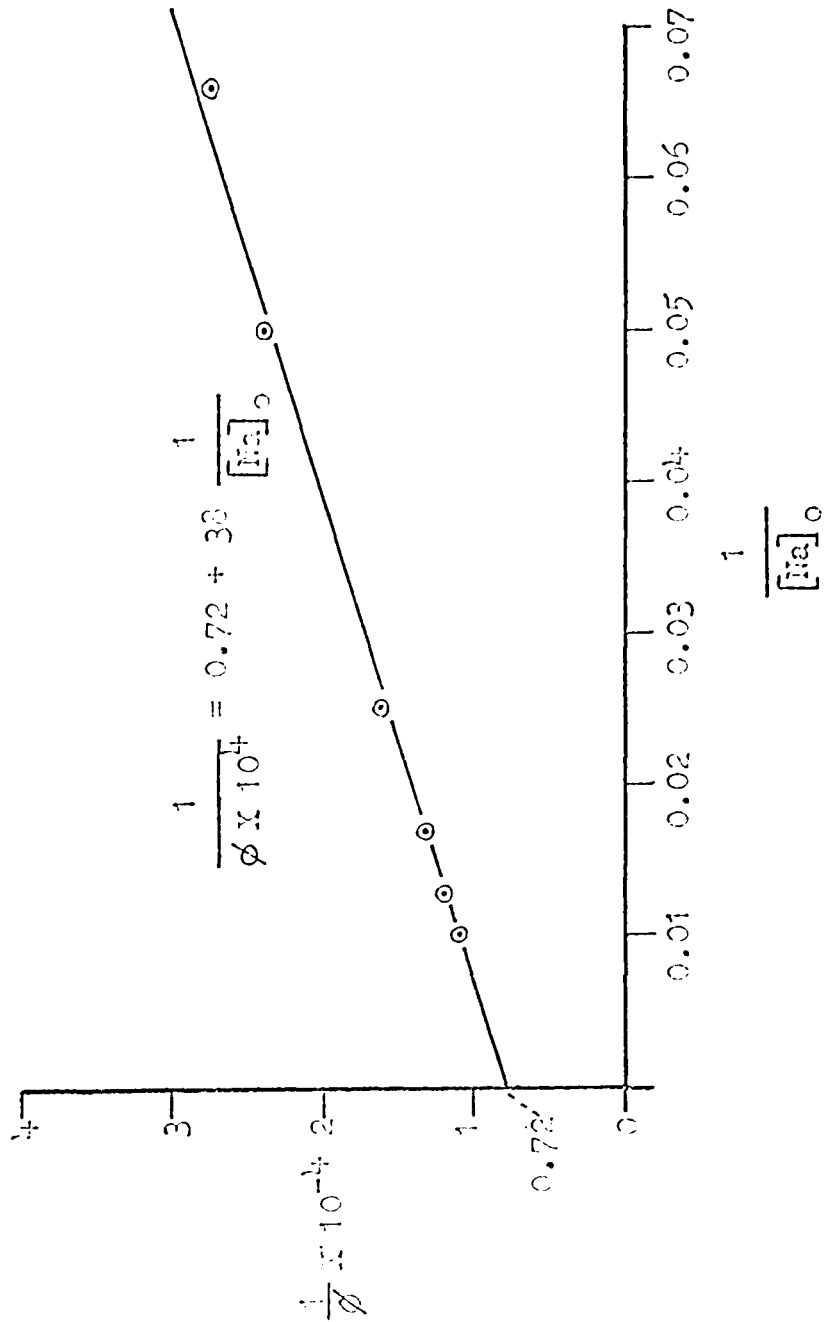


FIGURE 17

Relationship between P_D and $[Na^+]_o$

P_D is the sodium permeability coefficient in the Dainty-House layer of the skin (page 29 of text). P_D is expressed in cm/sec, $[Na^+]_o$ in $\mu\text{Eq/ml}$.

$$\frac{1}{P_D \times 10^5} = 0.05 + 0.067 [T_a]_0$$

A Present data

Cerejido et al. $(1/P_D \times 10^{-5})$

B

$[T_a]_0$

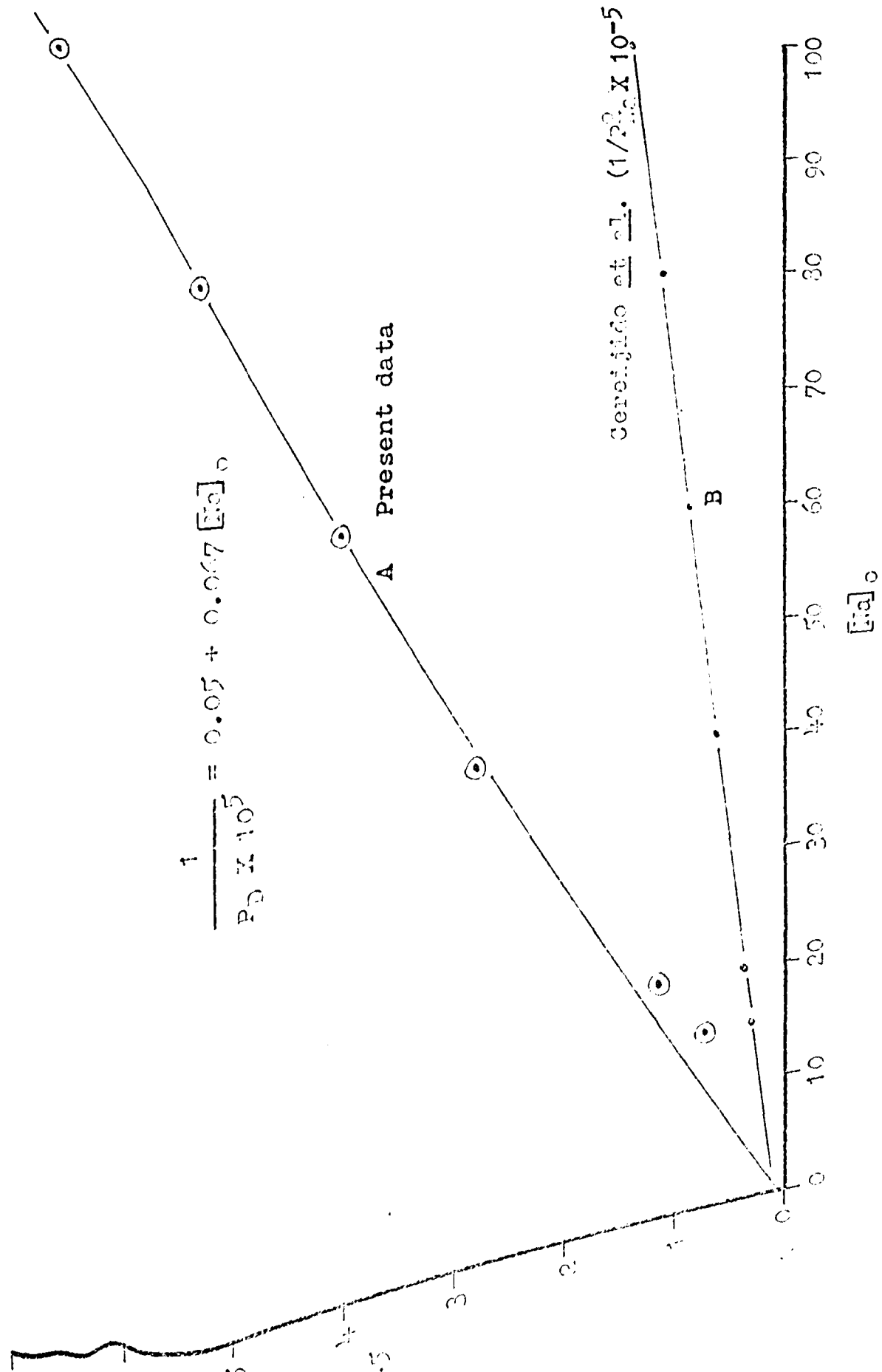


FIGURE 18
Plot of Equation (19)

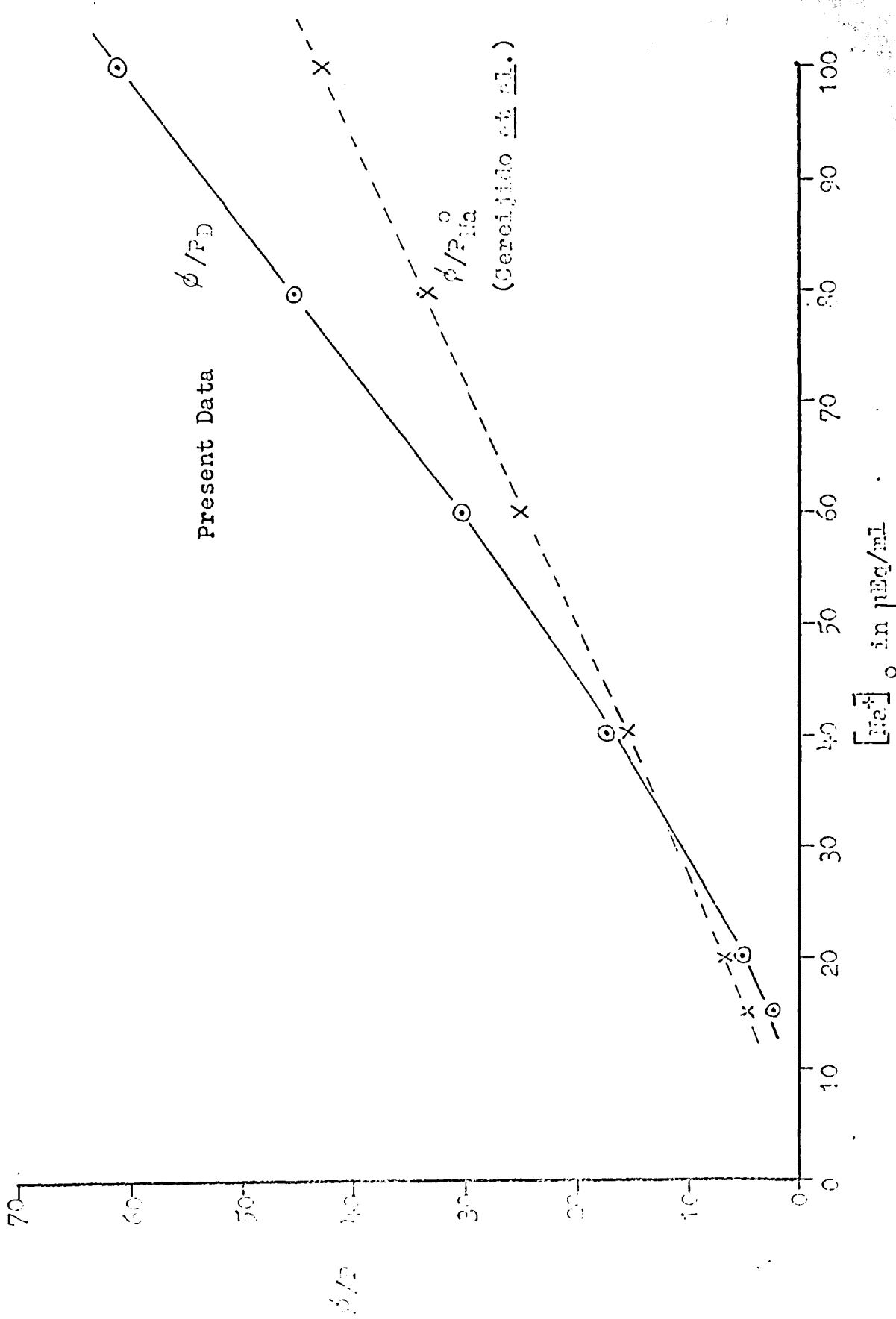


FIGURE 19
Plot of ΔV_{ob} against $[Na^+]_o$.

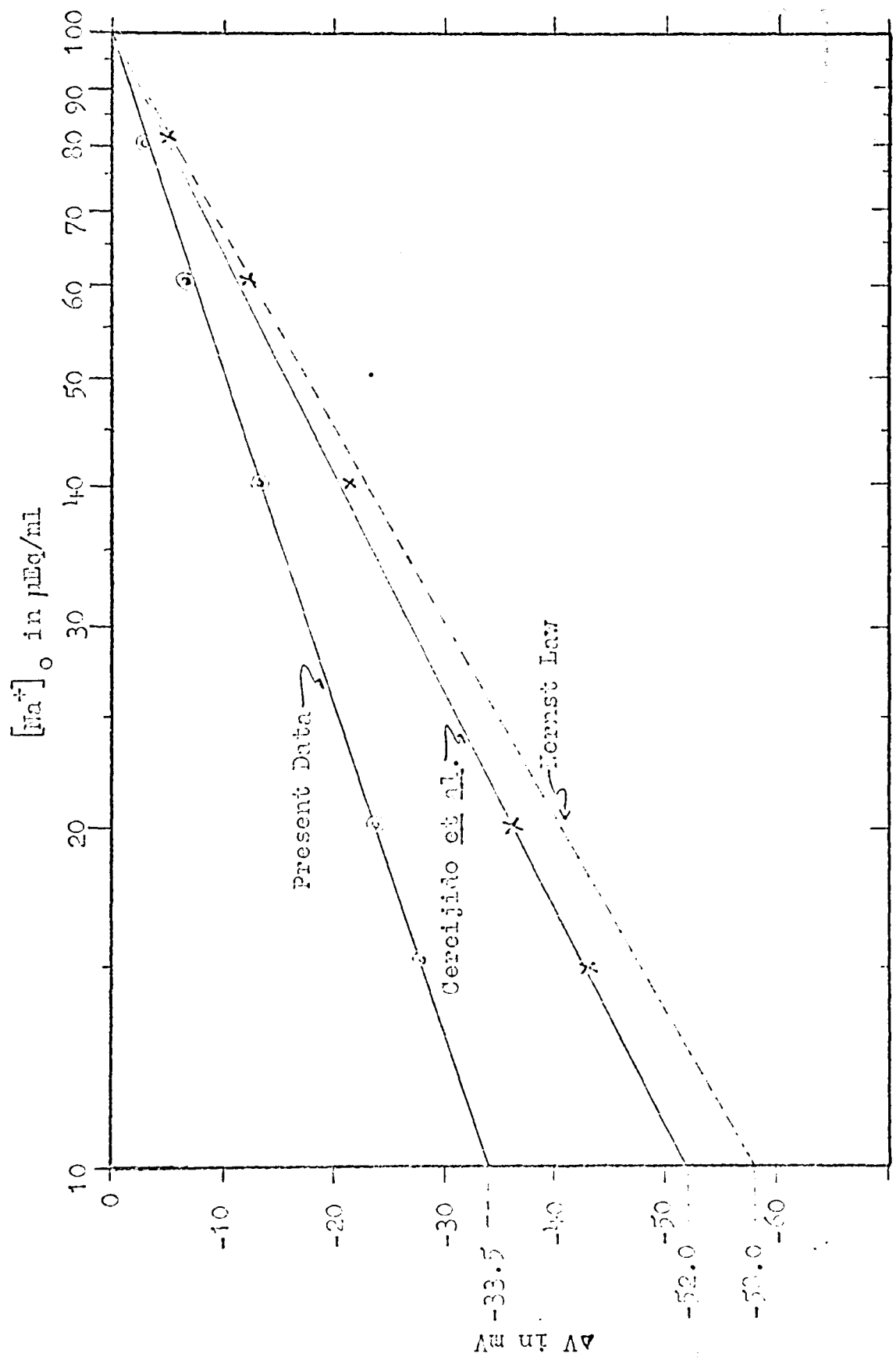


TABLE 1

Over-all sulfate permeability (P_{SO_4}) of frog skin obtained
from S^{35} measurements

P_{SO_4} cm/sec	DIRECTION OF FLUX	FROG SPECIES	REFERENCE
2.6×10^{-6}	outflux	?	Ballien and Schoffeniels (1961)
0.3×10^{-8}	influx	<u>R. pipiens</u>	Klahr and Bricker (1964)
0.4×10^{-8}	outflux	<u>R. pipiens</u>	Klahr and Bricker (1964)
0.5×10^{-8}	influx (?)	<u>R. pipiens</u>	Cereijido and Curran (1965)
4.2×10^{-7}	influx	<u>R. temporaria</u>	Ussing and Windhager (1964)
$P_{Na} =$ 0.4×10^{-6}	outflux	<u>R. pipiens</u>	Winn et al. (1964)

TABLE 2

Preparation of Ringer's solutions from stock solutions

One liter solutions were made,
pH was adjusted with H_2SO_4 .

SOLUTION no.	FINAL CONCENTRATION mM/l		ml of STOCK 0.5 M/l Na_2SO_4	ml of STOCK 0.5 M/l K_2SO_4	ml of STOCK 1.0 M/l SODIUM ISETHIONATE	ml of STOCK 0.5 M/l THAM	pH
	Na	K					
1	110	10	110	10	-	10	8.0
2	90	30	90	30	-	10	8.0
3	60	60	60	60	-	10	8.0
4	10	110	10	110	-	10	8.0
5	120	-	120	-	-	10	8.0
6	120	-	-	-	120	10	8.0
7	120	-	120	-	-	10	9.0
8	120	-	120	-	-	10	7.0
9	120	-	120	-	-	10	6.0

TABLE 3

Response of the epidermis to varying $[\text{Na}^+]_o$.
 pH 8.0, temperature 24-27 C, sulfate Ringer's

$[\text{Na}^+]_o$ μEq/mL	$[\text{K}^+]_o$	ELECTRICAL RESPONSE OF THE EPIDERMIS (P.D. in mV)			
		Group I Avg. 6 ♂ frogs	Group II Avg. 5 ♀ frogs	Group I + II Avg. ♂ + ♀	Group III Avg. 5 ♂ + ♀
110	10	93±4 *	51±10	77±9	91±4
60	60	94±2	49±9	72±8	61±3
30	90	83±2	42±8	64±8	51±3
10	110	67±1	34±7	52±6	43±2
110	10	99±3	49±10	76±9	88±2

* Standard error of the mean

TABLE 4
Sodium ion concentration in Na-isethionate solution
pH adjusted with HCl

By calculation using formula weight	By method of flame- photometry	By method of Na ⁺ electrode
mg/liter 120	mg/liter 125	mg/liter 107

TABLE 5

Electrical response of the epidermis to Na-isethionate ions as compared to sulfate ions.

Solution	$[\text{Na}^+]_0$ μEq/ml	Thin P.D., mV
$\text{Na}_2\text{SO}_4/\text{K}_2\text{SO}_4$	110	37±8
Na-isethionate	107	64±8
$\text{Na}_2\text{SO}_4/\text{K}_2\text{SO}_4$	110	35±6

TABLE 6

Effect of epinephrine on skin P.D. and response of the epidermis to change in $[Na^+]_o$ at pH 8.0

$[K^+]_o$ uEq/ml	$[Na^+]_o$ uEq/ml	Average Skin potential mV
110	10	16±4
90	30	17±4
60	60	17±4
10	110	13±3
110	10	19±5

TABLE 7

Dependence of Na⁺ flux (ϕ) and Na⁺ permeability coefficient (P_D) on [Na⁺]_o

1	2	3	4	5	6	7	8
PRESENT DATA					DATA OF GERRALDINO et al.		
[Na] _o μEq/ml	$\phi \times 10^4$ μEq × cm ⁻² × sec ⁻¹	C _x μEq/ml	P _D × 10 ⁶ cm × sec ⁻¹	ϕ/P_D	$\phi \times 10^4$ μEq × cm ⁻² × sec	P _{lin} ^o × 10 ⁶ cm × sec ⁻¹	ϕ/P_{lin}^o
15	0.36	12.3	16.4	2.2	1.56	34.0	4.6
20	0.42	15.2	3.3	4.8	1.81	27.3	6.5
40	0.61	23.1	3.6	17.0	2.50	16.5	15.2
60	0.75	29.5	2.5	30.0	2.36	11.6	24.7
80	0.86	35.1	1.9	45.3	3.03	9.1	33.4
100	0.92	40.2	1.5	61.3	3.11	7.4	42.1

LIST OF EQUATIONS

$$V = \frac{RT}{nF} \ln \frac{C_1}{C_2} \quad (1)$$

$$V = 58 \log \frac{[Na^+]_1}{[Na^+]_2} \quad (2)$$

$$V_{ob} = 58 \log \frac{[Na^+]_o}{[Na^+]_c} \quad (3)$$

$$V_{ob} = \frac{RT}{F} \ln \frac{P_{Na} [Na^+]_o + P_K [K^+]_o + P_H [H^+]_o + P_{SO_4} \sqrt{[SO_4^{2-}]_c}}{P_{Na} [Na^+]_c + P_K [K^+]_c + P_H [H^+]_c + P_{SO_4} \sqrt{[SO_4^{2-}]_o}} \quad (4)$$

$$V_{ob} = 58 \log \frac{P_{Na} [Na^+]_o}{P_{Na} [Na^+]_c + P_{SO_4} \sqrt{[SO_4^{2-}]_o}} \quad (5)$$

$$\beta = \frac{V_1 - V_2}{\log [Na^+]_1 / [Na^+]_2} \quad (6)$$

$$V_{ob} = 35 \log \frac{[Na^+]_o}{[Na^+]_c} \quad (7)$$

$$V_{ob} = 58 \log \frac{C_x}{[Na^+]_c} \quad (8)$$

$$\phi = \frac{[Na^+]_o - [Na^+]_c}{\frac{1}{P_L} + \frac{1}{P_D} + \frac{1}{P_{ob}}} \quad (9)$$

$$\phi = \frac{[Na^+]_o - C_x}{1/P_D} \quad (10)$$

$$P_D = \frac{\phi}{[Na^+]_o - C_x} \quad (11)$$

$$\frac{1}{\phi \times 10^4} = 0.72 = 38 \frac{1}{[Na^+]_o} \quad (12)$$

$$C_x = [Na^+]_c^{(1-k)} \times [Na^+]_o^k \quad (13)$$

$$\frac{1}{P_D \times 10^5} = 0.05 + 0.067 [Na^+]_o \quad (14)$$

$$\frac{1}{P_{Na}^o \times 10^5} = 0.03 + 0.013 [Na^+]_o \quad (15)$$

$$C_x = \frac{[Na^+]_o P_D - \phi}{P_D} \quad (16)$$

$$V_{ob} = 58 \log \frac{[Na^+]_o P_D - \phi}{[Na^+]_c P_D} \quad (17)$$

$$\Delta V_{ob} = 58 \log \frac{[Na^+]_o^{II} - (\phi/P_D)^{II}}{[Na^+]_o^I - (\phi/P_D)^I} \quad (18)$$

$$\phi/P_D = \frac{10 A [Na^+]_o + 10 B [Na^+]_o^2}{a [Na^+]_o + b} \quad (19)$$

LIST OF SYMBOLS

- A.....0.05 (Equation 19).
- a.....0.72 (Equation 19).
- B.....0.067 (Equation 19).
- b.....38 (Equation 19).
- βSlope factor of the electrical response line when the $[Na^+]_o$ is changed. β is defined by Equation (6).
- C_x Na^+ concentration in the solution near the outward face of the "outer border."
- d_LThickness of the unstirred fluid layer adjacent to the epidermis (cm).
- F.....Faraday's constant, 96,500 coulombs per gram equivalent.
- k.....35/58 or 0.604 (Equation 13).
- ln.....Natural logarithm ($2.3 \times \log_{10}$).
- n.....The valency of the ion species (Equation 1).
- P.....Permeability coefficient in cm/sec.
- P_{Na}^o Na^+ permeability coefficient of the "outer border" as defined by Cereijido et al. The corresponding symbol used in this thesis is P_D .
- ϕNet rate of Na^+ flux in $\mu Eq \times cm^{-2} \times sec^{-1}$.
- R.....The gas constant, 8.3 joules per degree per mole.
- T.....The absolute temperature.
- V.....Potential difference across a membrane.
- V_{ob}Potential difference across the "outer border" of the frog skin epidermis.

ΔV_{ob} ...Change in potential difference across the "outer border" when the sodium concentration is changed from one sodium concentration to another, $[Na^+]^I$ to $[Na^+]^{II}$.

Subscripts

- cPertaining to the intracellular fluid.
- DPertaining to the outer layers of the epidermis.
- LPertaining to the unstirred fluid layer adjacent to the epidermis.
- oPertaining to the solution on the epidermal side of the skin.
- obPertaining to the "outer border."

VITA

James Henry Martin, III was born March 31, 1943 in New Orleans, Louisiana. His primary education was divided between New Orleans and Windsor, New York. In 1956 he moved to Waynesboro, Virginia where he entered Waynesboro High School. He participated in an accelerated program for college bound science students. He was one of the twelve high school students selected to participate in the first Science Workshop sponsored by E. I. DuPont in Waynesboro. In June, 1961 he was graduated from Waynesboro High School, and in September he entered the University of Virginia. While studying at the University, he became active in the Westminster Fellowship and was elected treasurer and later Vice-President. He was selected House Manager for Westminster House, a Presbyterian owned house for college students in Charlottesville. He received the B. A. degree in biology in June, 1965. In September, 1965 he entered the University of Richmond. While at the University of Richmond, he was elected to Beta Beta Beta Honorary Biological Society, and he was awarded a Williams Fellowship. He received the degree of Master of Science in August, 1967.

Mr. Martin was awarded a teaching assistantship at the University of Tennessee where he will continue his graduate studies toward a Doctor of Philosophy Degree in September, 1967.