FLUXES OF Na^+, Cl^- AND WATER ACROSS THE "SHORT-CIRCUITED" PIGEON CROP MEMBRANE

Thesis for the Degree of M. S.

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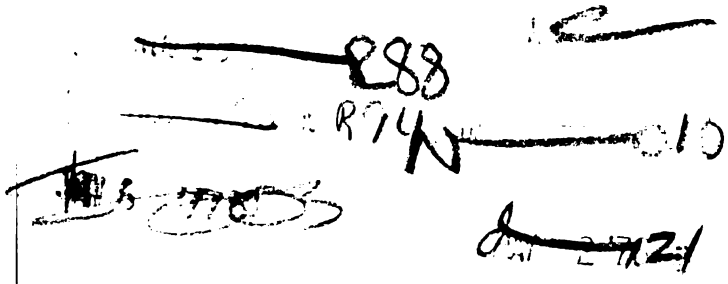

ABSTRACT

FLUXES OF NA^+, CL^- AND WATER ACROSS THE "SHORT-CIRCUITED" PIGEON CROP MEMBRANE

by Richard C. Rose

A study of the history of physiology indicates a close association of bioelectrical phenomena with the ionic flux characteristics of biological membranes. For example, the electrogenic activity of amphibian skins results from a preferential movement of ions through the skin.

In the present study the electrogenic activity of the pigeon crop membrane was investigated to determine if it results from a preferential ion movement through the membrane in a manner similar to that of the amphibian skin.

The muscle-free crop membrane was held in a Ussing-type chamber while the potential difference and short-circuit current across the membrane were alternately monitored. Under steady-state conditions, a solution of tritiated water with either Na^{22} or Cl^{36} was introduced into one side of the chamber and hourly samples were taken from the opposite chamber for 3-9 hours.

Although there was a wide variability in the potential difference (17.4 ± 16.5 mV), short-circuit current (20.3 ± 13.2 μA) and total resistance (0.86 ± 0.57K ohms) between membranes, the net Na^+ flux through each membrane could always be equated to the short-circuit current developed by

the membrane. Many of the characteristics demonstrated by ion pumps of other biological membranes were also demonstrated to exist in the crop membrane. It is postulated that the crop membrane possesses an ion pump mechanism capable of moving Na^+ from mucosa to serosa against an electro chemical gradient.

The ratio of the potential difference to the short-circuit current for each membrane was found to be an index of the permeability of that membrane to water and ions.

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By

Richard C. Rose

A THESIS

Submitted to
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Dedicated

to

my Mother

and

my Father

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CHAPTER I

INTRODUCTION

Fundamental to all living processes is the interaction of molecules, atoms and ions. Gilbert (1965) suggested that the first form of life resulted from the random collisions of molecules under the proper environmental conditions. The future of life was dependent on the ability of organisms to control the internal concentrations of the various molecular species operational in the living processes. Every living cell, for instance, requires specific internal concentrations of the various ions to continue functioning.

Membranes serve the cell, and the organism as a whole, in the selective entry of molecules by passive diffusion. A membrane may also function, with an investment of metabolic energy, in an active process to create the necessary chemical gradients between the intracellular and extracellular fluids.

An investigation of the permeability characteristics of a membrane is facilitated when the environmental conditions of the membrane can be controlled. For this purpose Ussing and his co-workers developed an _in vitro_ perfusion system with which they were able to maintain sheets of tissue from cold blooded animals (frogs and toads) in a viable state long enough to measure accurately ion fluxes.

The present problem was to determine if the electrogenic activity of the pigeon crop membrane results from a preferential movement of ions. No technique described in the literature has been successfully used in ion perfusion studies on sheets of tissue from homeotherms; the author adapted the Ussing-type perfusion system to serve this purpose. The author's data were used to determine that the passive permeability of a crop membrane can be predicted on the basis of the electrogenic properties of that membrane, a technique thought to be general enough to have application to other membranes of similar structure and properties.

CHAPTER II

REVIEW OF THE LITERATURE

The earliest investigation of electrical properties of biological membranes is attributed to Luige Galvani who discovered the electrical property of excised tissue by observing muscle spasms in frog legs suspended by copper hooks from an iron rod in 1791. DuBois-Reymond (1948) was the first to observe that the frog skin maintains a potential difference between the inside and outside. In 1892 Reid postulated that the skin of some amphibians could help maintain the salt and water balance of the animals by secreting NaCl inward. This could not be attributed to osmosis because it occurred when a piece of skin separates two identical Ringer's solutions.

In 1904 Galeotti made the important discovery, which was consistent with Reid's postulate, that Na^+ was needed to maintain the skin potential. Galeotti incorrectly postulated, however, that the potential was due to a preferential but passive permeability of Na^+ from the outside to the inside of the frog skin. Francis (1933) found that electrical current was generated for many hours by the frog skin and attributed this to the movement of salts by active transport (energy dependent transfer of a solute against an electrochemical gradient.)

Huf (1935) found that Cl^- moved from the outside solution to the inside solution of the *in vitro* frog skin bathed on both sides with identical Ringer's solution. Krogh (1937) further demonstrated the movement of Cl^- through the frog skin. He depleted the body Cl^- of frogs by exposing them to a stream of distilled water and then measured a Cl^- uptake of 0.1 $\mu eq/hr/cm^2$ from a solution of only 1 mM Cl^-. The rate of uptake increased as the external Cl^- concentration increased.

Nernst developed the following equation which describes the potential difference generated by an ion concentration difference between two solutions separated by a membrane:

$$E = \frac{RT}{zF} \ln \frac{c_1}{c_2} \qquad \text{(equation 1)}$$

where:

E = potential difference across membrane (mV)

R = gas constant (8.317 joules/mole-K^o)

T = temperature (degrees K)

z = valence

F = Faraday's constant (96,500 coulombs/eq.)

c_1 = ion concentration of inside solution

c_2 = ion concentration of outside solution

Ussing (1949) modified the basic Nernst equation to describe the ratio of diffusion rates of an ion between two solutions of electrical difference E′:

$$E' = \frac{RT}{zF} \ln \frac{M_{in}}{M_{out}} \qquad \text{(equation 2)}$$

where:

M_{in} = ion flux inward $\qquad$ M_{out} = ion flux outward

Using this formula Ussing demonstrated that I^- and Cl^- passively diffuse through the frog skin but the movement of Na^+ cannot be explained in terms of diffusion. Huf and Parish (1951) replaced part of the internal Cl^- concentration with phosphate ion and found that the rate of movement of Na^+ across the frog skin was unaltered. They postulated that the potential difference across the frog skin was due to an active (energy consuming) movement of Na^+. The ground work was thus laid for the classical experiment of Ussing and Zerahn (1951) in which they showed that Na^+ was the only species actively moved through the perfused in vitro frog skin.

Ussing's Short-circuit Technique

The circuitry used by Ussing was essentially the same as that used in the present experiment, shown in Figure 4. The external (non-membrane) circuit was completed by a microammeter and a voltage supply (consisting of a battery and a variable resistor) connected in series with the platinum electrodes. During the experiment the applied e.m.f. was adjusted by the variable resistor such that the potential

difference across the skin, as read from the potentiometer, was maintained at zero. It is assumed that as each Na^+ crossed the membrane it was neutralized by an electron from the external circuit. Consequently, the current in the external circuit (short-circuit current, s.c.c.) was a measure of the rate of Na^+ accumulation in the inside solution. The movement of Na^+ (in μeq) was 105% of the s.c.c. (in μeq e^-) rather than the theoretical value of 100%. The slight discrepancy was attributed to diffusion effects.

Ussing found that K^+ could not be substituted for Na^+ in the outside solution; the s.c.c. was reduced but was still equal to the amount of Na^+ transported when this change was made. Only Li^+ was able to be substituted for Na^+. When Li^+ was added to the outside solution the s.c.c. was larger than the amount of Na^+ transported, indicating that the new ion species was able to support some current (Ussing, 1954).

The Na^+ pump mechanism appears to be dependent on the presence of K^+ in the inside solution. Fukuda (1942) found that if a K^+-free solution was used in the inside solution, the potential difference dropped very rapidly almost to zero. Harris and Maizels (1951) showed that under these circumstances the active transport of Na^+ was reduced. Rb^+ was found to be able to replace K^+ almost completely and Cs^+ replaced it partially.

Anatomy of the Frog Skin

The frog skin consists of a mesodermal chorion and an ectodermal epithelium. The chorion contains blood vessels and connective tissue whereas the epithelium is made up of tightly packed cells of the stratum germinativum and one or more layers of cells undergoing keratinization. Ottoson et al. (1953) have shown by electron microscopy that the germinativum is bordered by a basement membrane which is thought to represent a separation of electrical charge between the corium and the germinativum.

Anatomy of the Pigeon Crop Membrane

Dumont (1965) has used light and electron microscopes in a histological study of the pigeon crop membrane. He reports the luminal surface of the crop membrane has a stratified squamous epithelium approximately twelve cells thick which is adjacent to a thin glycocalyx. The lamina propria is located beneath the glycocalyx and is composed of connective tissue, blood vessels and nerves. A transverse and a longitudinal layer of smooth muscle fibers is located external to the lamina propria. A thin serosa covers the entire organ.

Beams and Meyer (1931) described the epithelium of the crop in terms of two layers on a functional basis. They called the superficial layer lining the lumen of the crop the "nutritive layer." The stratum is 8-10 cells thick in the non-brooding bird and become 2-3 times thicker when

the young hatch. The deeper or basal cells of the epithelium were referred to by Litwer (1926) as the "proliferating epithelium." A cross section of the crop membrane with the lamina propria and smooth muscle removed is shown in Figure 1.

Mechanism of Sodium Transport

Figure 2A illustrates the properties of the epithelial cell currently thought to account for its ion pumping ability. Koefoed-Johnsen and Ussing (1958) postulated a high permeability to Na^+ and moderate permeability to Cl^- of the outward-facing membrane of the germinal epithelial cell along with a very low permeability to K^+. The inward-facing membrane has a high permeability to K^+ and Cl^- but a low permeability to Na^+. If, as they postulated, the pump mechanism is located on the inward facing membrane it is easily explained that NaCl is moved from outside to inside and the K^+ concentration inside the cell remains high. Studies with different osmolarities of Na^+ and K^+ salts were done to confirm the permeabilities as described above.

Figure 2B illustrates the carrier-based transport scheme that accounts for many of the facts known about the Na^+-K^+ pump. Hokin and Hokin (1960) studied the role phosphatidic acid plays in the Na^+ pump activity of avian salt glands. On the assumption that Na^+ is the actively transported ion in the salt gland as it is in the frog skin and nerve axon, they found a correlation between the amount of

Figure 1.--Cross-section of a pigeon crop membrane.

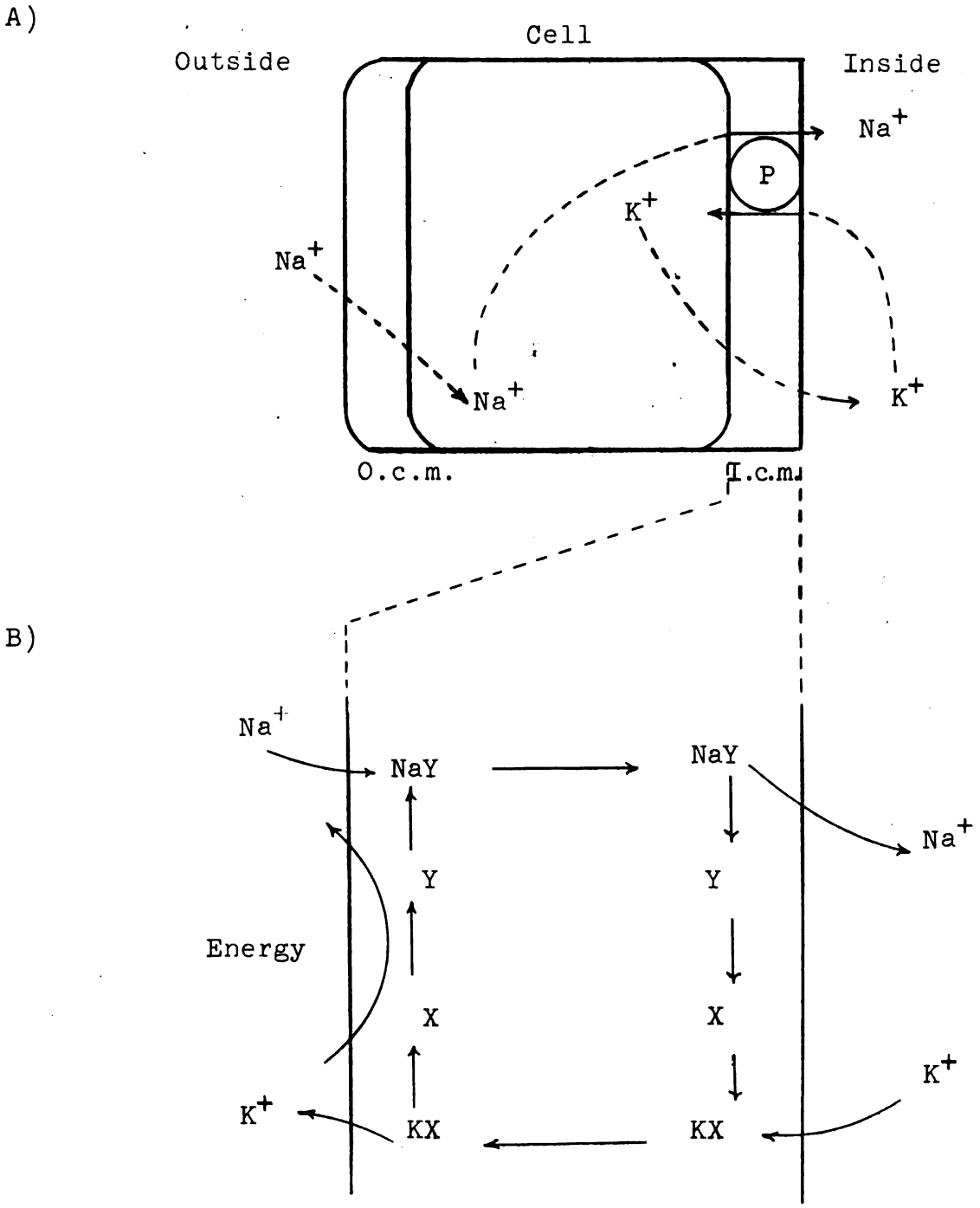


Figure 2.--Ion pumping system illustrated on the cellular and membrane level.

Figure 2A illustrates the ion pumping system of the epithelial cells as described by Ussing. The outward-facing membrane (O.c.m.) is permeable to Na^+ and the inward-facing membrane (I.c.m.) is permeable to K^+. The pump (P) actively transports Na^+ from the cell and K^+ into the cell. Figure 2B illustrates the carrier-based transport mechanism; X and Y represent the carrier molecules.

Na^+ pumped and the turnover rate of phosphatidic acid. They postulated a phosphatidic acid-diglyceride cycle capable of carrying Na^+ from one surface of the membrane to the other. The enzymes necessary for catalysis of the reactions of this cycle have been found in the membrane fraction.

Metabolism

Francis and Gatty (1938) suggested that NaCl movement through the frog skin was dependent on metabloism since 1 mM cyanide, a known metabolic inhibiter, abolished the net flux. Bromo-acetate reduced the net inward Cl^- movement which was restored with the addition of pyruvate or lactate.

Huf and Parish (1951) reasoned that an increase in metabolic substrates to the frog skin should increase the net flux of NaCl. However, when they added the sodium salts of ATP or glycerophosphate to the inside solution of the perfused frog skin there was no increase in the rate of movement of Na^+. They assumed the increased Na^+ gradient counteracted any increase in Na^+ transport.

Zerahn (1956) and Leaf and Renshaw (1957) compared oxygen consumption rates with the active transport of Na^+ in the frog skin. Zerahn used the Winkler method for O_2 determination. He compared O_2 consumption of the normal perfused membrane with that of a skin in a Na^+-free solution (inhibited Na^+ pump activity). Leaf and Renshaw used the polarographic method with vibrating electrodes to measure oxygen consumption. They used posterior pituitary hormone

to stimulate Na^+ transport, and then compared the increases in Na^+ transport and O_2 consumption. Both experiments indicated that 1 equivalent of O_2 was consumed for each 16-20 equivalents of Na^+ transported. Leaf and Renshaw placed the skin in an oxygen-free Ringer's solution to determine whether any transport would occur from anaerobic metabolism. The Na^+ transport decreased to 10% of its original value within 30 minutes.

The following factors were reported by Zerahn (1956) to affect Na^+ transport and oxygen consumption:

1. Na^+ concentration in the outside solution
2. Potential difference across the skin
3. Oxygen tension
4. Time of year
5. Species of the animal
6. Temperature
7. Additions of posterior pituitary hormone
8. Method of blank determination

Metabolic inhibitors have been reported to reduce the potential difference and the short-circuit current of the frog skin and toad bladder. Schoffeniels (1955) found that dinitrophenyl (DNP) caused the frog skin potential to fall and finally to reverse. In the inactivated membrane, Na^+ movement is passive and with Ringer's solution on each side of the membrane there is no net flux.

The presence of ouabain reduces the frog skin potential difference (Koefoed-Johnsen,1957). This was reported to be

due to its inhibitory effect on ATPase activity (Schatzman, 1953). Schatzman suggests that in the Ussing model of the Na^+-K^+ pump ouabain has an inhibitory action at the site for K^+ activation near the outside surface of the cell membrane but does not compete with Na^+ at its activating site on the inside surface of the cell membrane.

ATPase Activity

Consistent with the theory that the Na^+ pump activity is dependent on energy derived from oxidative phosphorylation, Skou (1957) found in crab nerve a Na^+ and K^+ dependent ATPase necessary for Na^+ and K^+ transport.

Post et al. (1960) found an insoluble ATP-splitting enzyme system in human erythrocytes. The activities of the enzyme system and the Na^+ pump had the following group of properties in common:

1. Both were located in the membrane.
2. Both utilized adenosine triphosphate in contrast to ionsine triphosphate.
3. Both required Na^+ and K^+ together. Either Na^+ or K^+ alone was ineffective.
4. K^+ activation was competitively inhibited by high concentration of Na^+ in both systems.
5. Ammonium ion substituted for K^+ but not for Na^+ in both systems.
6. Oubain inhibited both systems.

Farquhar and Palade (1966) have studied the localization of ATPase activity in the epidermis of Rana pipiens, Rana catesbiana and Bufo marinus. They incubated skin sections in a Wachstein-Meisel medium (0.83 mM ATP, 10 mM $MgSO_4$, 2.4 mM $Pb(NO_3)_2$) and then treated each section with $(NH_4)_2S$ to convert the ATPase reaction product (lead phosphate) into black lead sulfide which could be seen with an electron microscope. They found the enzyme present in all cell membranes that face the labyrinth of epidermal extracellular spaces. No activity is indicated in the outer and inner fronts of the epidermis.

From their results Farquhar and Palade suggest modifications be made on the Koefoed-Johnsen and Ussing model (1958) of the frog skin membrane. The recently proposed model allows the membrane a larger surface area for pumping activity. The geometry of the extracellular spaces may help restrict the diffusion of "captured" Na^+ towards the interstitial fluid. Koefoed-Johnsen and Ussing suggested that the outward facing membrane, permeable to Na^+ but not to K^+, was localized on the outer aspect of the s. germinativum. Farquhar and Palade suggest that this membrane should be located nearer the outer front of the epidermis since there appears to be no structurally continuous barrier on the inner side of the s. germinativum as Koefoed-Johnsen and Ussing suggested. Farquhar and Palade propose that the pump mechanism is located in all cell membranes facing the

extracellular spaces. The most likely area for free diffusion of K^+ is the inward facing membrane of the epidermis.

Studies done on the development of the p.d. across the frog membrane may help to determine whether the theory of Farquhar and Palade is more accurate than that of Koefoed-Johnsen and Ussing. Engback and Hoshiko (1957) inserted a micro-electrode into the frog skin from the outside and found that the potential across the skin was established in two steps. Immediately upon entering the skin the electrode recorded a negativity in relation to the outside medium. This was assumed to be the resting potential of a superficial epithelial cell. A positive potential of about 60 mV appeared at a depth of 50μ . The full potential of the membrane was recorded when the micro-electrode reached a depth of about 100μ .

The results of Engback and Hoshiko appear to be more consistent with the theory of Farquhar and Palade than that of Koefoed-Johnsen and Ussing. The two step development of the p.d. may be due to the diffuse distribution of the Na^+ pumps as described in Figure 3.

Hormonal Action on Membranes

The neurohypophyseal hormones (ADH) have been found to alter the rate of movement of salts and water across many biological tissues. ADH increases the Na^+ pump activity of the frog skin (Fuhrman and Ussing, 1951) and the permeability of the skin to Na^+ (Ussing, 1955). The s.c.c. of

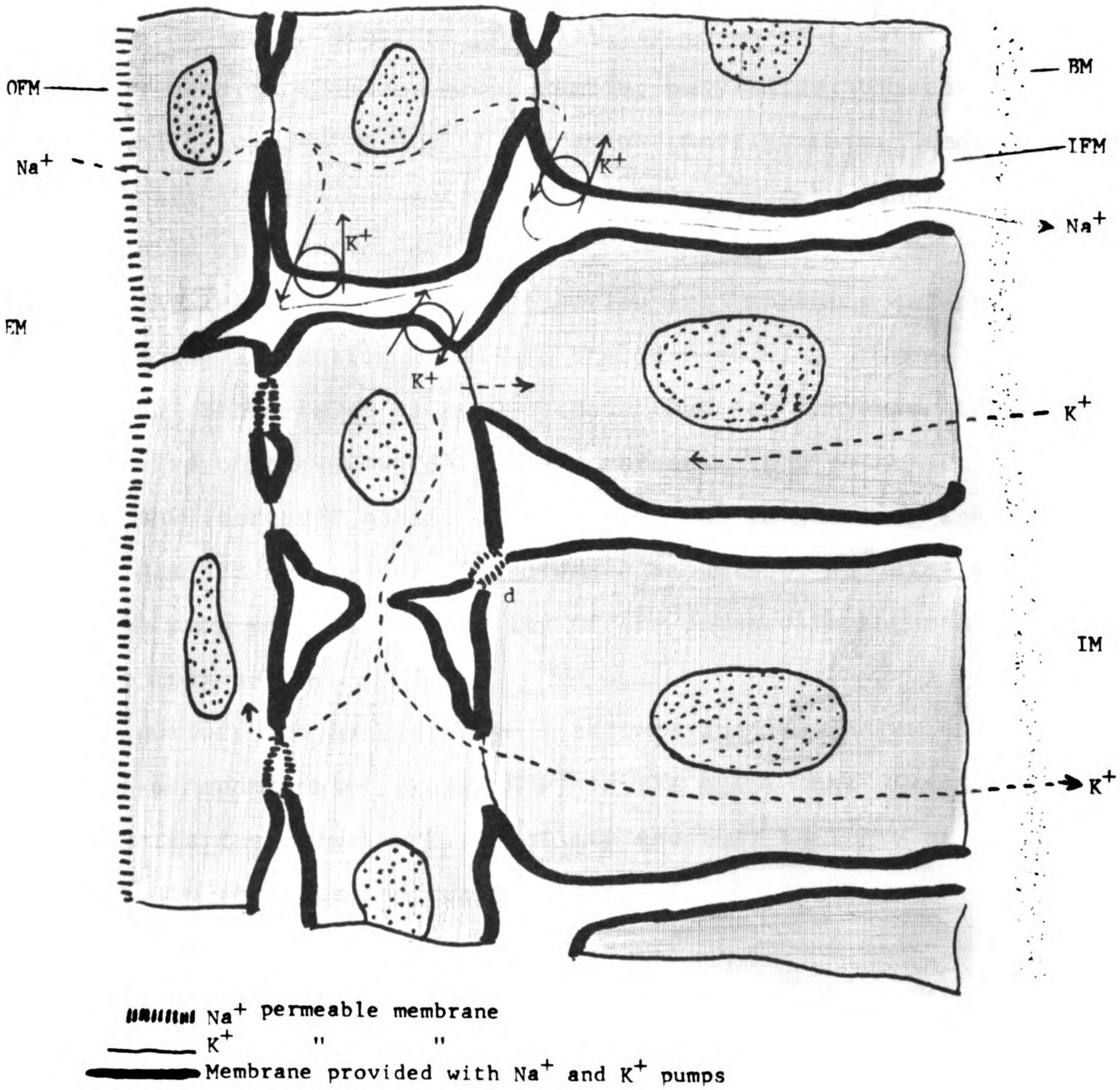


Figure 3.--Model of amphibian epidermis.

This figure depicts the modified model of amphibian epidermis by Farquhar and Palade (1966). The three cell layers represent schematically, from left to right, the s. corneum, s. spinosum, and s. germinativum. The intercellular space is shown in white and the intracellular space in gray. The nuclei appear in strippled gray. EM, external medium; OFM, outward facing membrane; IFM, inward facing membrane; BM, basement membrane; IM, internal medium; d, desmosome.

the toad bladder is increased (Leaf, Page, and Anderson, 1959), as is the membrane permeability to water (Leaf, 1960), urea, acetamide, propionamide, butyramide, dimethyl formamide, cyanamide and nicotinamide (Maffly, Hays, Lamdin and Leaf, 1960) although the permeability to many other molecules is unaffected.

At least two different theories are currently defended concerning the action of ADH on the permeability of membranes. Schwartz et al. (1960) hold that ADH increases the pore size of the outward-facing membrane. They point out that the increased active transport of Na^+ in the frog and toad bladder may be due to increased amounts of Na^+ available to the pump as the permeability of the outward-facing membrane increases.

Orloff and Handler (1961) believe that adenosine 3',5'-monophosphate (cyclic AMP) is the agent that directly regulates the membrane permeability and that the role of ADH is to regulate the concentration of cyclic AMP.

Crabbe (1960) found a stimulatory effect of aldosterone on the toad bladder similar to that of ADH on the frog skin but his system required an incubation time of about one hour before the effect became evident (Kleinzeller and Kotyk, 1960). Edelman, Bogoroch and Porter (1963) demonstrated that in the toad bladder aldosterone is located in the nucleus of the epithelial cells and acts by promoting DNA-dependent RNA systhesis that produces proteins involved in the coupling of metabolism with Na^+ transport.

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

White King pigeons of both sexes, one to seven years old, were purchased within two weeks of their use from the Cascade Squab Farm, Grand Rapids, Michigan. There was no attempt to regulate the amount of light hours the birds received each day; the present experiments were done during the summer months. The birds were kept in continuous supply of water and feed.

The birds were killed by cervical dislocation. The feathers over the crop area were plucked and then the skin was teased from the crop membrane. Two samples of membrane were obtained from each bird; each was held in an interlocking ring system two inches in diameter. The serosal surface and musculature of the membrane were separated from the mucosa under a dissecting microscope. The mucosal membrane (2.54 cm^2) was then placed between the halves of a lucite perfusion chamber. The operation took approximately ten minutes during which the membrane was constantly bathed in warm bird Ringer-glucose solution (see page 26).

Apparatus

Two types of lucite perfusion chambers were used to hold the membranes. The first type used was an electrically driven stirring device. Oxygen was released into an air space above the medium. The second type of block (Figures 4 and 5) used an air-lift siphon for stirring. This block system was first used by Knoll (1962) to hold frog skins and was later modified by Edelhauser for perfusion studies on trout corneas (1966). The block system was again modified by the author to make s.c.c.-p.d. readings across frog skin and pigeon crop membranes. Pressurized air was passed through an air purifier and flow equalizer (Koby Corporation, Melrose, Mass.) and then combined with 95% oxygen and 5% carbon dioxide. The resulting mixture (72% nitrogen, 27.5% oxygen, 0.5% carbon dioxide) was passed through a series of buffered wash bottles to saturate it with water. This mixture was used to operate the air-lift siphon and also supply the perfusion medium with oxygen and regulate its pH. Lucite caps were used to prevent evaporation and splashing of the perfusion medium. Both types of lucite perfusion chambers were found to furnish adequate stirring and oxygen to maintain the electrical activity of the membrane for up to twelve hours. The temperature of the medium was maintained at 38-40^{o} C.

Miniature calomel electrodes (E.H. Sargent & Co., Detroit, Michigan) were used to measure potentials in both perfusion systems. The saturated electrolyte in these

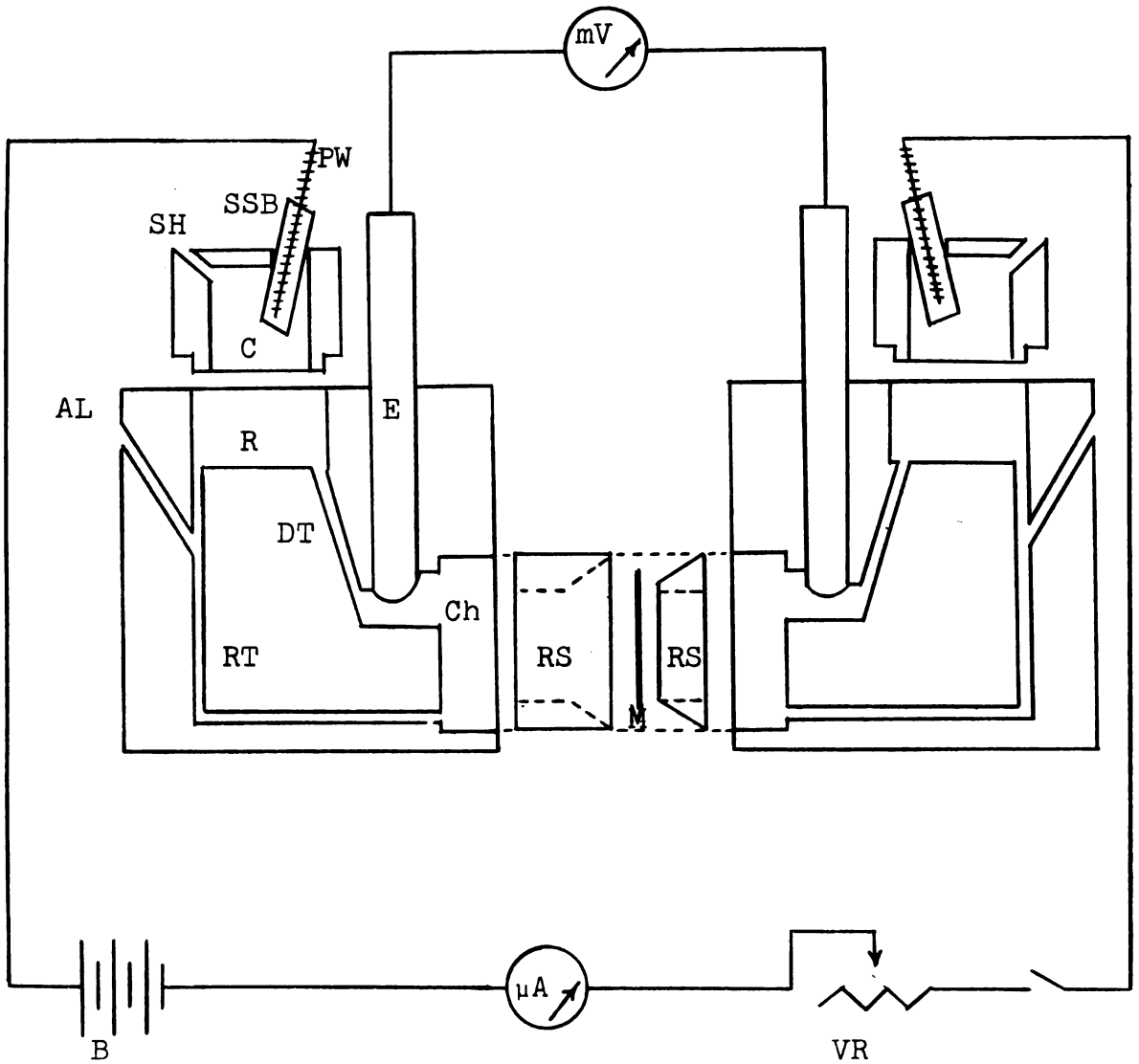


Figure 4.--Illustration of perfusion blocks and electrical circuits.

AL - Air line
B - Battery (1.5v)
C - Cap
Ch - Chamber
DT - Delivery tube for fluid
E - Electrode
M - Membrane
mV - Millivoltmeter (0-100v)
PW - Platinum wire
R - Reservoir
RS - Ring system
RT - Recovery tube for fluid
SH - Sample hole
SSB - Short-circuit bridge
VR - Variable resistor (o-11 x 10^6 Ohms)
μA - microammeter (o - 11 μA)

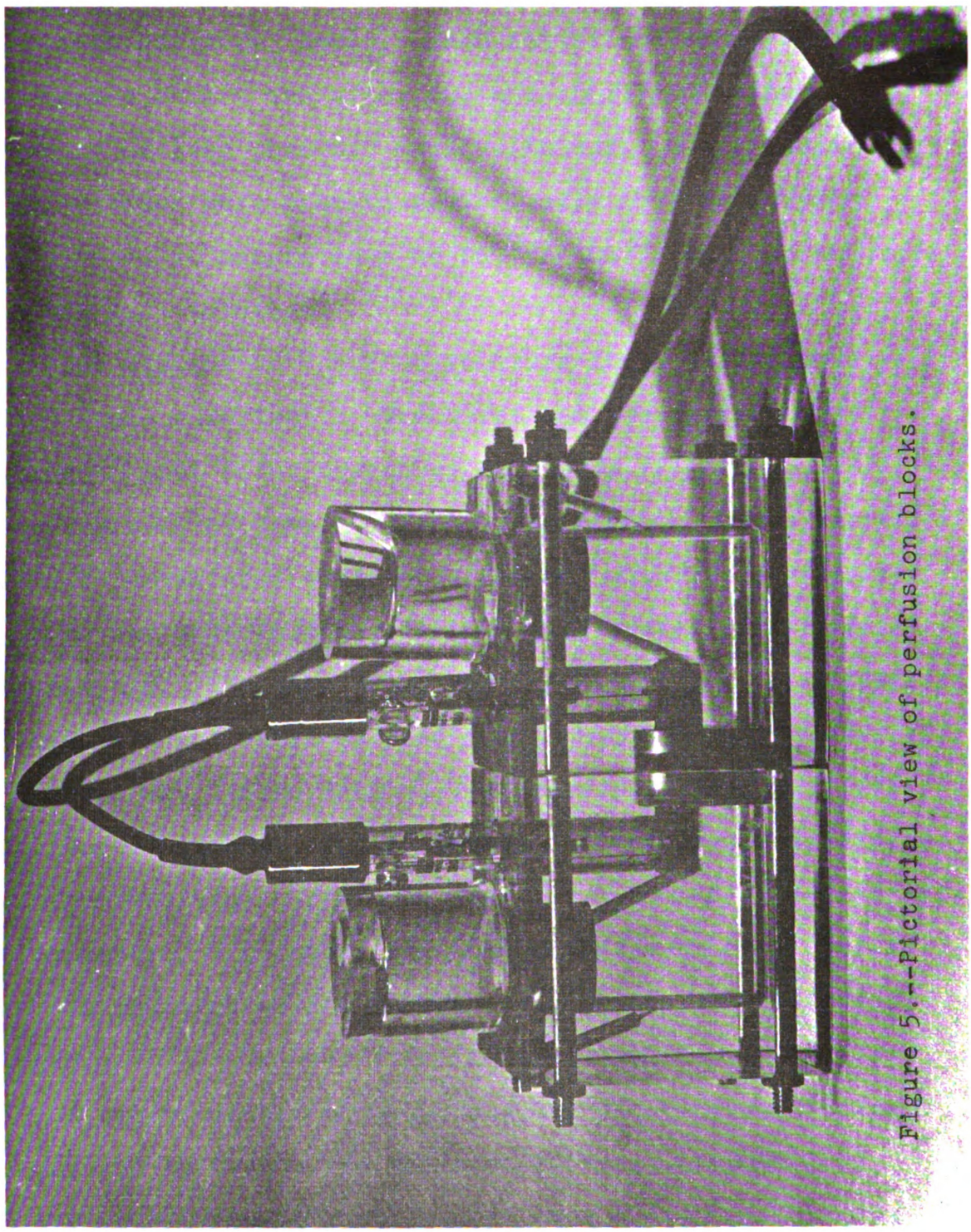


Figure 5.--Pictorial view of perfusion blocks.

electrodes was changed from KCl to NaCl because the K^+ released by the electrodes was found to alter the membrane potentials after 2-3 hours. Each set of converted electrodes was checked before and after use and confirmed to have an asymmetry of less than 0.3 millivolts and 1.0 microampere. The electrodes were checked periodically in a NaI crystal well counter to insure that no Na^{22} or Cl^{36} had been absorbed into the NaCl solution.

Two systems for short-circuiting the membrane were used in this experiment. The first was the manual type similar to that first described by Ussing (Figure 4). A counter-e.m.f. of three 1.5 volt batteries in series was connected to a variable resistor. Contact with the perfusion solution was made through platinum wire seated in a Ringer-agar mixture. The external resistance was adjusted so that the membrane potential as read from a recording electrometer (YSI model 81, Yellow Springs Instrument Co., Antioch, Ohio) was reduced to zero. The exogenous current required to maintain the potential difference equal to zero was read from a microammeter. A constant adjustment of the external resistance was required as the membrane's potential or resistance changed.

A second method of recording the short-circuit current was devised by Frantz and Holland (1967, in preparation) and was used in these experiments. The p.d. is constantly nulled to zero by a Sargent model SR recorder converted to a cathode follower and calibrated to record 4.0 mV full

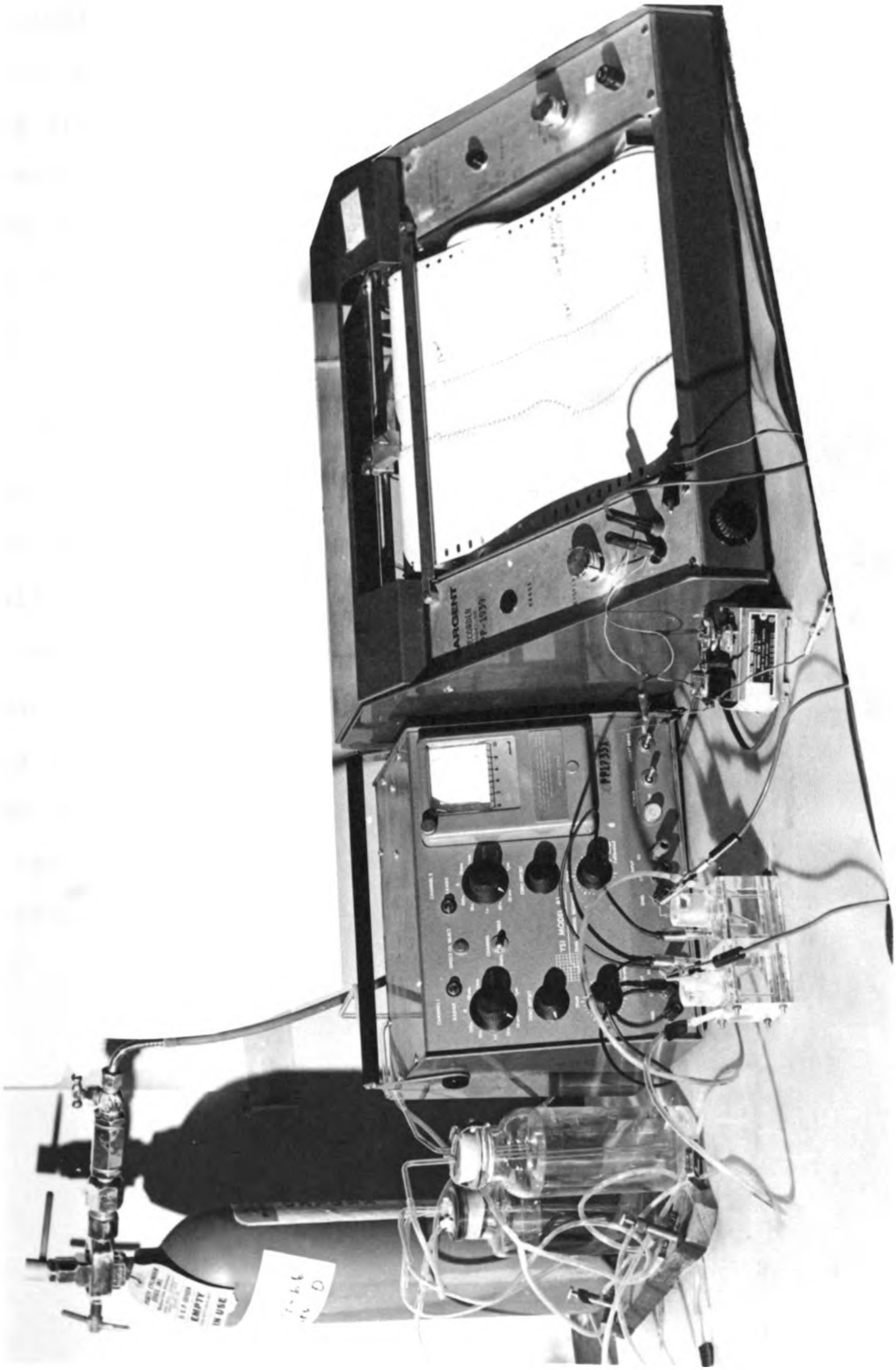
scale. When a 40K ohm resistor is intermittently introduced into the circuit by a timing device, the s.c.c. is read directly off the chart as 1 μA/scale unit. This method of recording the s.c.c. eliminates the need for manual adjustment of the external resistance.

The perfusion apparatus was surrounded by grounded copper wire screening to prevent spurious magnetic or electrical impulses from affecting the p.d. and s.c.c. measurements. The volumes, and therefore the hydrostatic pressures, of the bathing solutions on the two sides of the membrane were maintained constant to prevent changes in the p.d.

After the perfused membrane reached a condition of steady p.d. and s.c.c. 100 μl of a radioisotopic solution containing tritiated water (HTO) with either $Na^{22}Cl$ or $NaCl^{36}$ was added to the mucosal or serosal solution. Two samples, one at 3 min and one at 10 min, were taken from the inoculated solution using 20 μl micropipettes (Drummond microcaps, Drummond Scientific Co., Broomall, Pa.). Hourly (20 μl) samples were taken from the opposite solution. At the end of each experiment the perfused section of the membrane was put in a counting vial *in toto*.

Each counting vial (Packard Instruments Co. Inc., Downers Grove, Ill.) contained 15 ml of a scintillation solution, composed of a primary scintillator (5 gm PPO, 2.5-diphenyloxazole), a secondary scintillator (50 mg a-naphthylphenloxanole), 80 gm napthalene and enough dioxane to make one liter. The HTO and Na^{22} or Cl^{36} content of

Figure 6.--Pictorial view of experimental apparatus.


ARGENT

each sample was assayed for 100 minutes in a Nuclear Chicago Mark I Liquid Scintillation Spectrometer. The counting efficiency was 30% for HTO and 90% for Na^{22} and Cl^{36}. The counting error was less than 2% on each sample. Each sample was corrected for background activity and quenching.

The bird Ringer's solution used in these experiments contained the following concentrations of ions:

Na^+	150.0 meq/l	Cl^-	128.0 meq/l
K^+	3.25 meq/l	SO_4 =	1.0 meq/l
Ca^{++}	2.20 meq/l	HCO_3^-	29.1 meq/l
Mg^{++}	2.50 meq/l	Glucose	5.55 meq/l

The K^+-free solution contained all the ions listed above with Na^+ substituted for K^+. The choline chloride bird Ringer's solution contained 150 meq/l choline and less than 5 meq/l Na^+.

Na^+, K^+ and Ca^+ concentrations were determined on a Coleman Model 21 flame photometer. Cl^- concentrations were determined on a Coleman microtitrator using the method of Shales and Shales (1947).

CHAPTER IV

RESULTS

I. Electrical Activity of the Crop Membrane

A potential difference exists between two identical Ringer's solutions separated by the crop membrane (Table 1). Because this membrane generates a cationic current *in vitro*, the serosal solution acquires a positive charge with respect to the mucosal solution. The electrogenic properties can be expressed in the Ohm's law relation, V = IR, adapted to the perfused membrane: V is the potential difference; I is the short-circuit current (equivalent to the cationic current); and R (total resistance of the circuit) can be calculated as p.d./s.c.c.

TABLE 1.--Average electrical properties of fifty crop membranes.

	n = 50
Potential difference	17.4 ± 16.5 mV
Short-circuit current	20.3 ± 13.2 μA
Total resistance	0.86 ± 0.57K ohms

II. Evidence for an Active Ion Pump in the Crop Membrane

The net Na^+ flux across the membrane as determined with Na^{22} accounts for all of the s.c.c. developed by the membrane. This is evidence for a Na^+ pump according to Ussing's theory (see Chapter II, p.5). Table 2 shows that after the membrane pumping mechanism becomes saturated with Na^{22} (4 hours) the amount of Na^+ (in μeq.) moved from the mucosal solution to the serosal solution is essentially equivalent to the s.c.c. (in μeq. e^-) developed by the membrane. The amount of Na^+ reaching the serosal solution is actually slightly greater than can be accounted for on the basis of the s.c.c. because there is a small diffusion of Na^+ across the membrane in both directions (see Results IV, p. 39). To account for this diffusion, Na^{22} was put in the serosal solution of three membranes and the diffusion rate from the serosal to the mucosal surface was measured (Table 2).

The methods used for calculating the amount of Na^+ crossing the membrane and the amount of s.c.c. during the same time period are as follows:

The assumptions made in these calculations are:

1. the tissue reacts the same to Na^{22} as to Na^{23};
2. the bathing medium is completely and instantaniously mixed by the stirring apparatus;
3. the injection of the isotopic Na^+ does not produce a significant Na^+ gradient.

TABLE 2.--Ion flux and short-circuit current.

Time after isotope injection	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	9 hrs.
Na^+ mucosal (μeq Na^+/cm^2)	0.39	0.87	1.38	1.70	2.08	2.56	2.80	3.90	4.53
to serosal (μeq e^-/cm^2)	0.67	1.06	1.42	1.69	2.00	2.24	2.52	3.78	4.08
Na^+ serosal (μeq Na^+/cm^2)	0.0	0.08	0.08	0.24	0.24	0.28	0.29		
to mucosal (μeq e^-/cm^2)	0.51	0.98	1.47	1.89	2.24	2.56	2.87		
Cl^- mucosal (μeq Cl^-/cm^2)	0.0	0.10	0.16	0.18	0.19	0.23	0.24		
to serosal (μeq e^-/cm^2)	0.34	0.67	1.00	1.33	1.65	1.89	2.05		
Cl^- serosal (μeq Cl^-/cm^2)	0.0	0.07	0.10	0.14	0.20	0.21	0.22		
to mucosal (μeq e^-/cm^2)	0.59	1.14	1.65	2.09	2.48	2.83	3.11		

The amount of current (s.c.c.) used to maintain the p.d. equal to zero is calculated from the Faraday law of electrolysis as:

$$\mu\text{eq of electrons} = \frac{[I\ (\mu A)][t\ (\text{sec.})]}{\text{Faraday}\ (\mu A\ \text{sec}/\mu\text{eq})}$$

$$\frac{[80\ \mu A][3600\ \text{sec.}]}{96500\ \mu A\ \text{sec}/\mu\text{eq}} = \underline{\underline{3\ \mu\text{eq of } e^-}}$$

During the same time interval the amount of Na^+ crossing the membrane is calculated as:

10,000 c.p.m. Na^{22} initially in mucosal solution

0 c.p.m. Na^{22} initially in serosal solution

600 μeq Na^+ in mucosal solution

600 μeq Na^+ in serosal solution

50 c.p.m. Na^{22} in serosal solution at time t

$$\left[\frac{\mu\text{eq } Na^+ \text{ in mucosal soln.}}{\text{initial c.p.m. } Na^{22} \text{ in mucosal soln.}}\right]\left[\begin{array}{l}(\text{c.p.m. } Na^{22} \text{ in serosal} \\ \text{soln. at time t})\end{array}\right]$$

$$= \mu\text{eq } Na^+ \text{ in serosal soln.}$$

$$\left[\frac{600}{10,000}\right]\left[50\right] = \underline{\underline{3\ \mu\text{eq } Na^+}}$$

The data of Table 2 is presented in graphic form in Figure 7. The straight line is a theoretical plot of 1 μeq of Na^+ moved across the membrane for each μeq of electrons

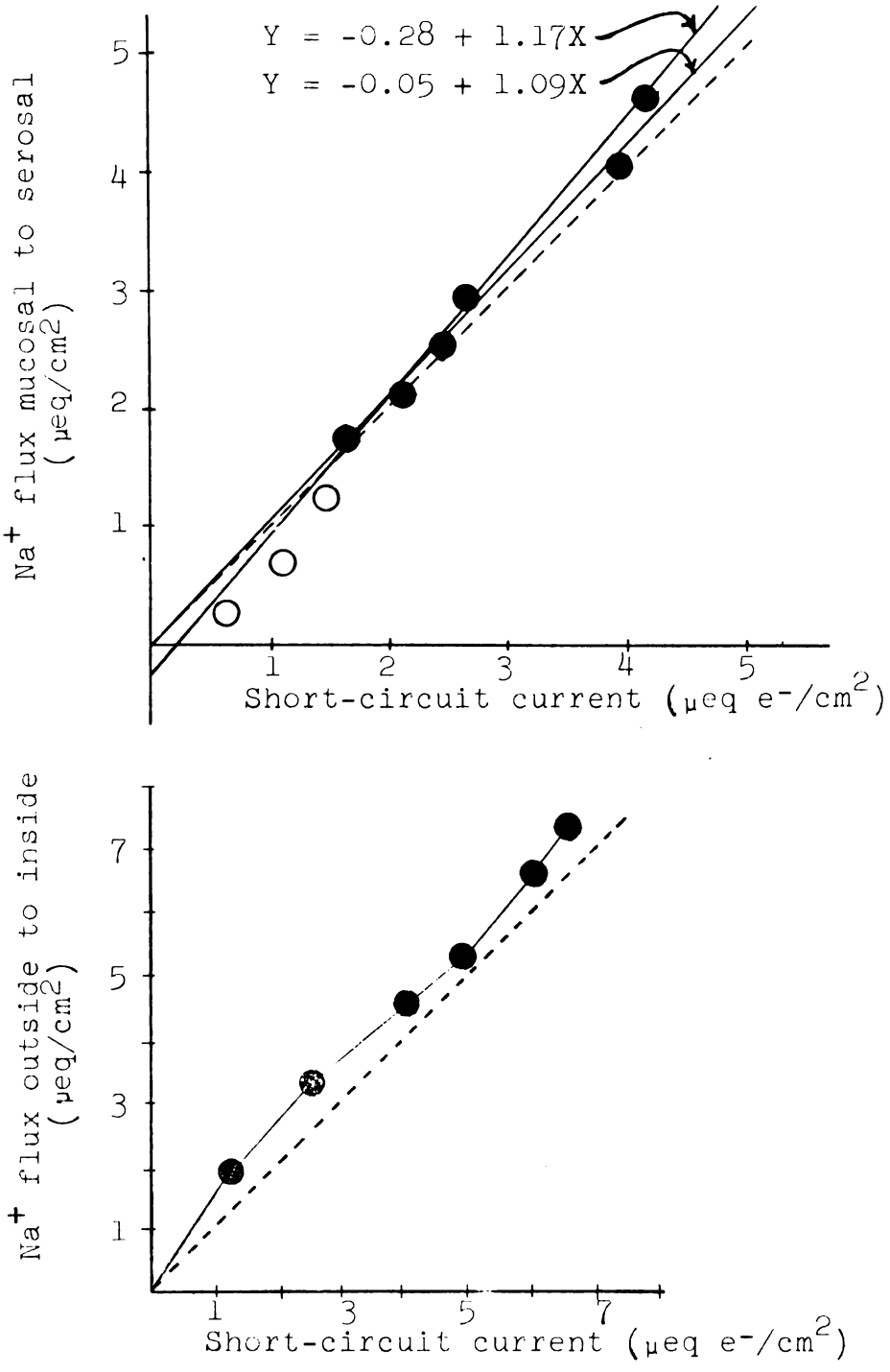


Figure 7.--Comparison of Na^+ flux and short-circuit current in pigeon crops and frog skins.

Upper: Average regression of Na^+ on e^- for four crop membranes. Dots represent hourly post saturation data where Y = -0.05 + 1.09X. Circles represent hourly pre-saturation data. For total nine hour data Y = -0.28 + 1.17X.
Lower: Average regression of Na^+ on e^- for two frog skins.

The theoretical line in each case is dotted, slope = 1.0.

from the exogenous current supply. In the first four hours of the experiment the amount of Na^+ moved from mucosal to serosal appears to be less than the theoretical amount; this is because the Na^+ pump is not immediately saturated with Na^{22}. After the four hour reading (post saturation), the calculated value for Na^+ movement from mucosal to serosal is slightly greater than the theoretical value because some Na^{22} diffuses through the membrane in addition to that being pumped through the membrane. While the gradient for Na^{23} is slightly negative in the mucosa to serosa direction, there is a positive Na^{22} gradient from mucosa to serosa. This causes an insignificant isotopic error.

For an average of four crop membranes the slope of the regression of μeq of Na^+ on μeq of e^- (s.c.c.) for 1-9 hours is 1.17 with the Y intercept at -0.28 (Figure 7). The correlation coefficient for this data is $r = 0.9944$, a highly significant correlation. The post saturation data (4-9 hours) for the same membranes has a regression slope of 1.09 with the Y intercept at -0.04. The correlation coefficient for the post saturation data is $r = 0.9905$.

A plot of Na^+ movement and s.c.c. versus time for three individual membranes (Figure 8) shows that when the ion pump activity is high (membranes 1 and 2) the amount of Na^+ moving from the mucosal solution to the serosal solution slightly exceeds the s.c.c. after four hours. When the ion pump activity is low (membrane 3) the membrane does not become saturated with Na^{22} and, therefore, little Na^{22}

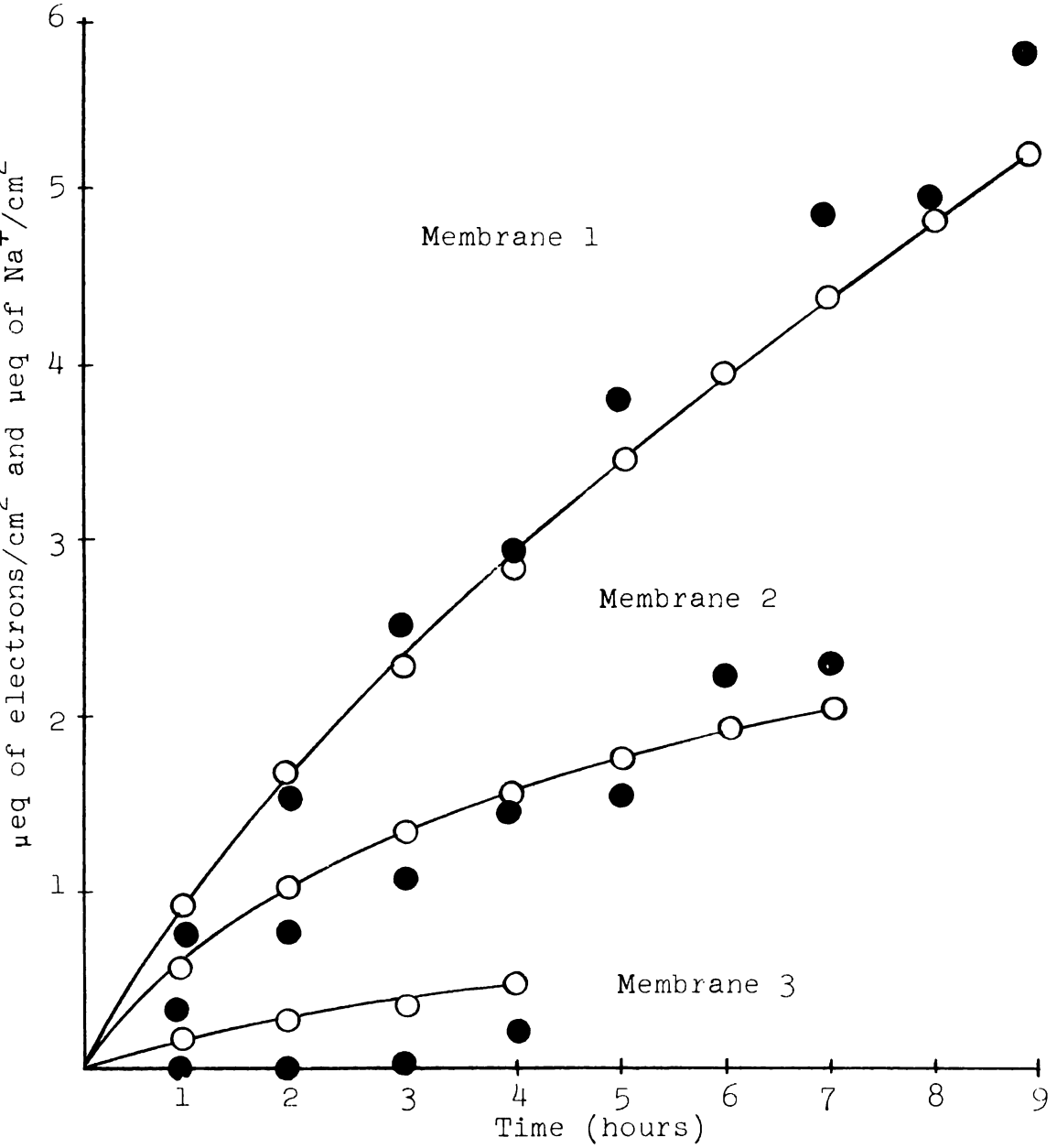


Figure 8.--Short-circuit current and sodium flux versus time. Short-circuit current indicated by circles; sodium movement indicated by solid dots.

reaches the serosal solution before the preparation begins to deteriorate.

A Na^+ pump must be postulated only to explain the movement of Na^+ in the direction mucosal to serosal. The amount of Na^+ moving from serosal to mucosal is seen to be small and independent of the s.c.c. (Table 2), and can be explained in terms of diffusion (p. 39). Likewise, the movement of Cl^- across the membrane in both directions is small and independent of the s.c.c., and is explained in terms of diffusion.

As a check on the methods used by the author, Na^+ movement from the outside to the inside of two frog skins was measured using the perfusion techniques described for the pigeon crop experiments. Figure 7 shows that the Na^+ movement from the outside to the inside surface of the frog skin is approximately 110% of the s.c.c. developed by the skin. This result is similar to that of Ussing's experiments (Chapter II, p. 5).

The Na^+ pumps of the frog skin (Steinback, 1937), erythrocyte (Glynn, 1957), muscle (Steinback, 1951) and other membranes are K^+ dependent ion pumps. The Na^+ pump of the crop membrane is also K^+ dependent. Figure 9 shows that when the usual bird Ringer's solution is replaced by a K^+-free Ringer's solution on the serosal side the p.d. increased briefly and then decays to zero. The transitory increase in the p.d. is believed to be due to a sudden outflux of K^+ due to the increased concentration gradient.

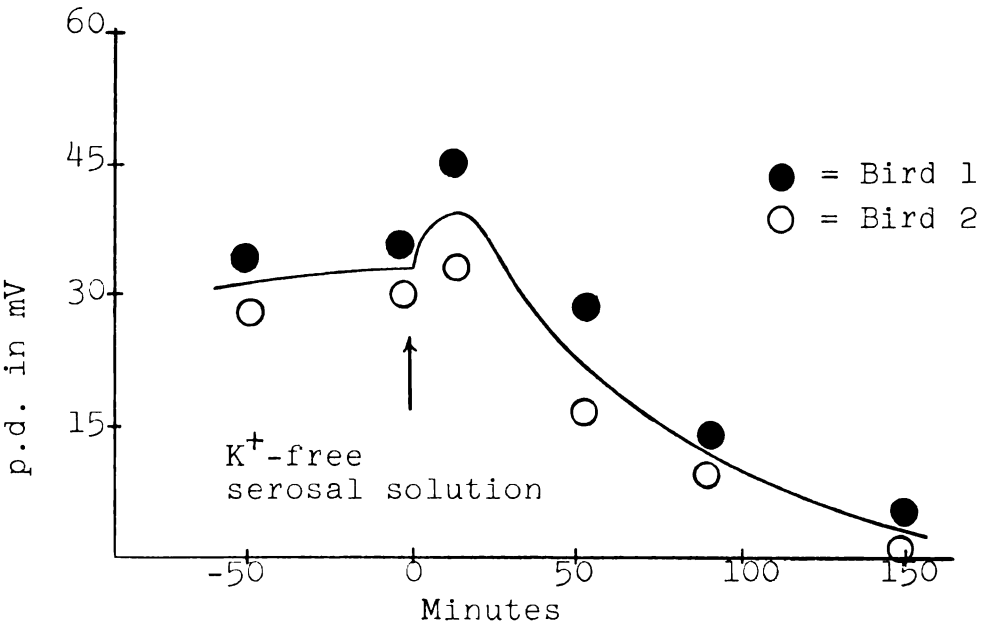


Figure 9.--Effect of a potassium-free solution on the potential difference.

Average of two crop membranes.

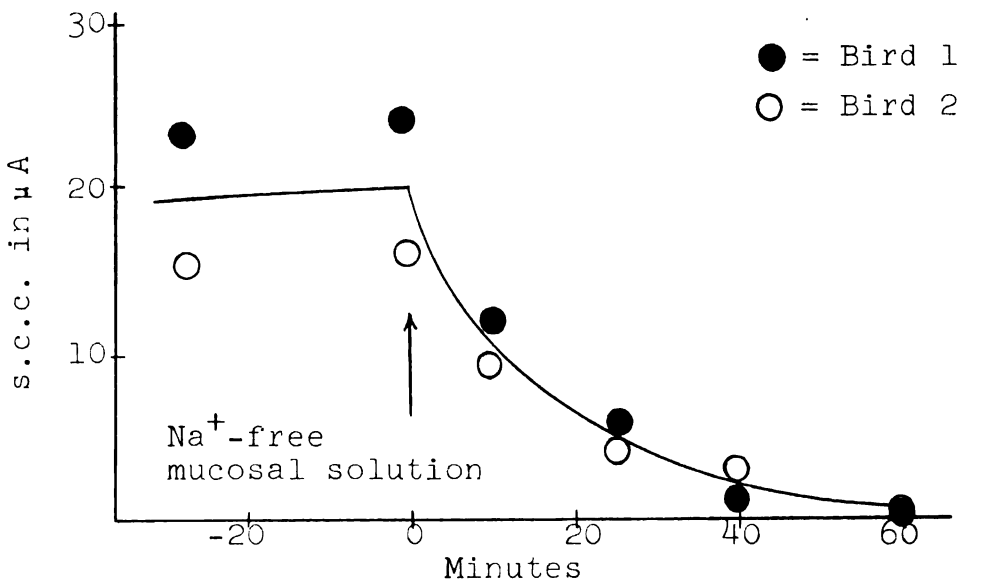


Figure 10.--Effect of a sodium-free mucosal solution on the short-circuit current.

Average of two crop membranes.

The gradual decrease of the p.d. demonstrates that the Na^+ pump is K^+ dependent.

If the s.c.c. developed by the crop membrane is due to an ion pump mechanism transporting Na^+ from the mucosal solution to the serosal solution, a replacement of choline for Na^+ in the mucosal solution should result in a lower s.c.c. as is shown in figure 10.

If the s.c.c. across a membrane is due to an active transport process, a deficiency of energy supply to the pumping mechanism should decrease the s.c.c. Two kinds of experiments demonstrate the dependence of the s.c.c. of the crop membrane on metabolic energy. When the usual bird Ringer's solution (containing glucose) is replaced by Ringer's containing no glucose, the s.c.c. steadily decreased. When an isosmotic glucose solution is added to the serosal solution the s.c.c. maintains a steady value or increased (Figure 11). DNP (10^{-4} M) reduces the s.c.c. to zero (Figure 12).

Ouabain is a competitive inhibitor of the Na^+ pump carrier enzyme in biological membranes (Schatzmann, 1953). Ouabain (10^{-4} M) in the serosal bathing solution of the crop membrane reduces the s.c.c. to zero (Figure 13).

A chemical process, whether in a living or non-living system, is expected to proceed at a faster rate at higher temperatures due to increased kinetic energy of the reacting molecules. The s.c.c. of the crop membrane, which is assumed

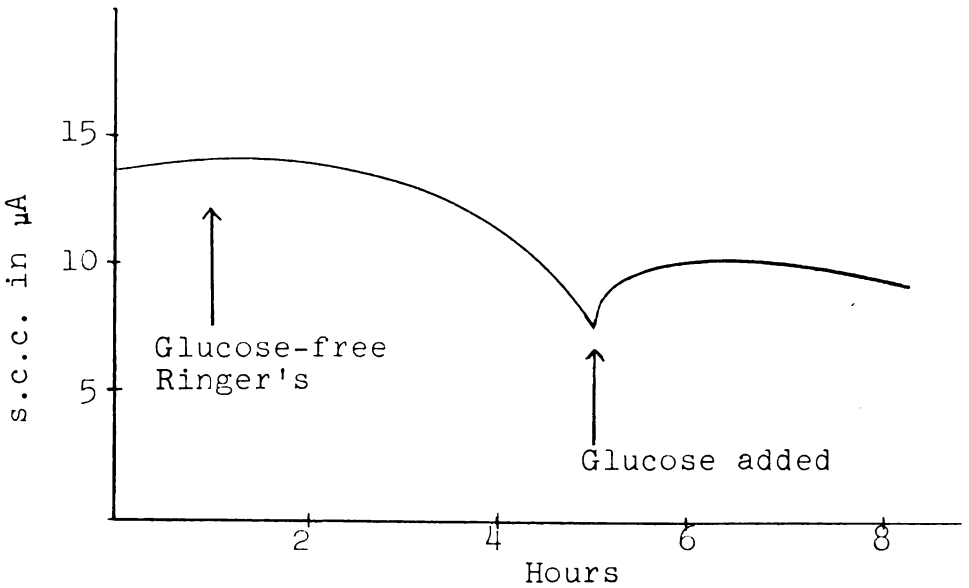


Figure 11.--Dependence of short-circuit current on glucose.

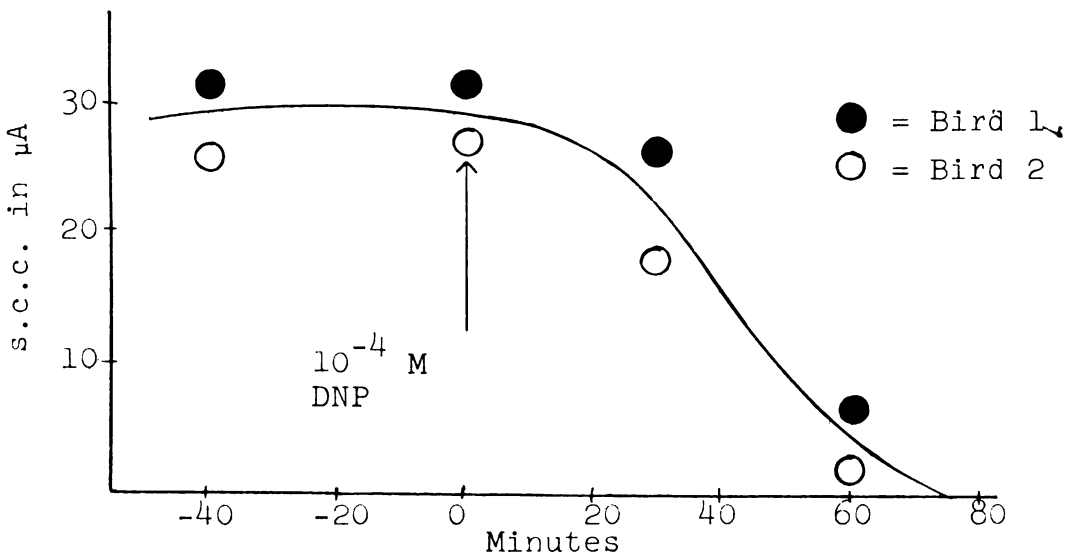


Figure 12.--Effect of DNP on the short-circuit current. Average of two crop membranes.

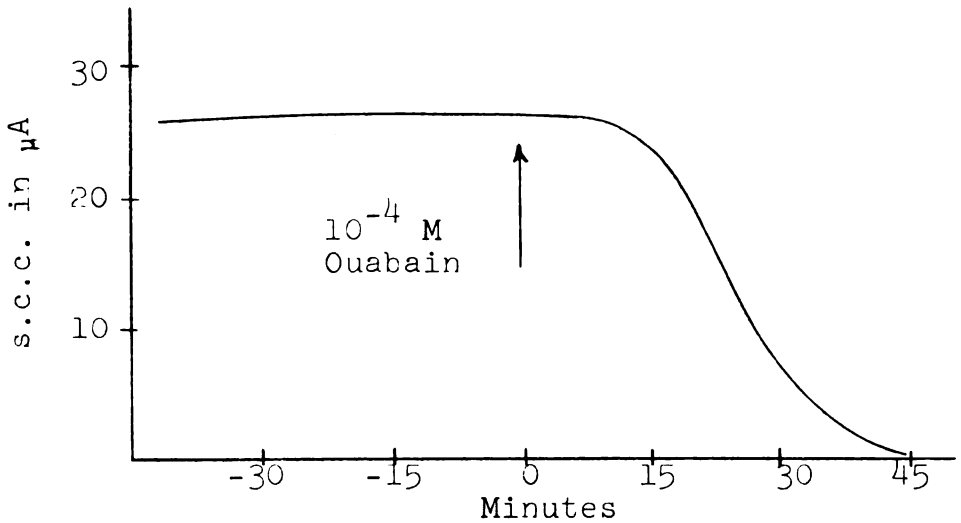


Figure 13.--Effect of ouabain on the short-circuit current.

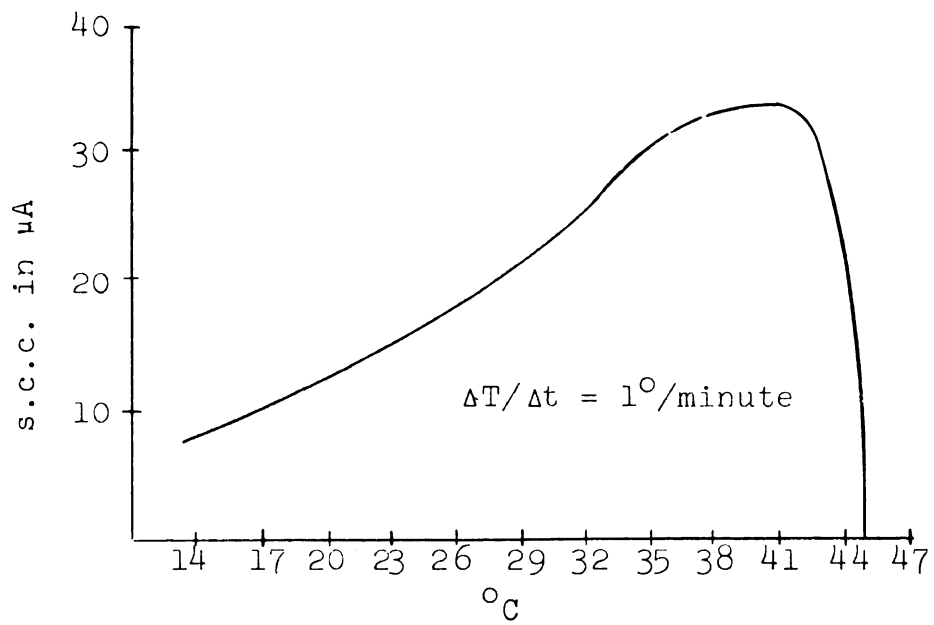


Figure 14.--Effect of temperature changes on the short-circuit current.

to be the result of several chemical processes, is demonstrated to be temperature dependent, as shown in Figure 14.

III. Retention of Na^+, Cl^-, and H_2O in the Crop Membrane

The present experiments have demonstrated that the crop membrane has an asymmetrical uptake of ions and water from the two bathing solutions (Table 3). The crop membrane retains 89% less Na^+, 88% less Cl^-, and 45% less water when the isotopes are introduced into the mucosal solution as opposed to the serosal solution. The different saturation ratio of Na^+, Cl^- and H_2O from the two sides of the membrane may be due to a functional permeation barrier in the membrane as described in the discussion section.

TABLE 3.--Membrane retention of ions and water.

	Na^+ μeq/mg	Cl^- μeq/mg	H_2O meq/mg
From mucosal soln.	0.007 ± 0.005	0.006 ± 0.003	0.007 ± 0.005
From serosal soln.	0.066 ± 0.034	0.054 ± 0.034	0.013 ± 0.012

IV. Prediction of Crop Membrane Permeability to Ions

Na^+ and Cl^- diffusion through the crop membrane can be predicted on the basis of the p.d./s.c.c. which will be referred to in this thesis as "internal ionic resistance = R_i." The passive ionic permeability and the internal ionic resistance are inversely related. Figure 15 shows that when

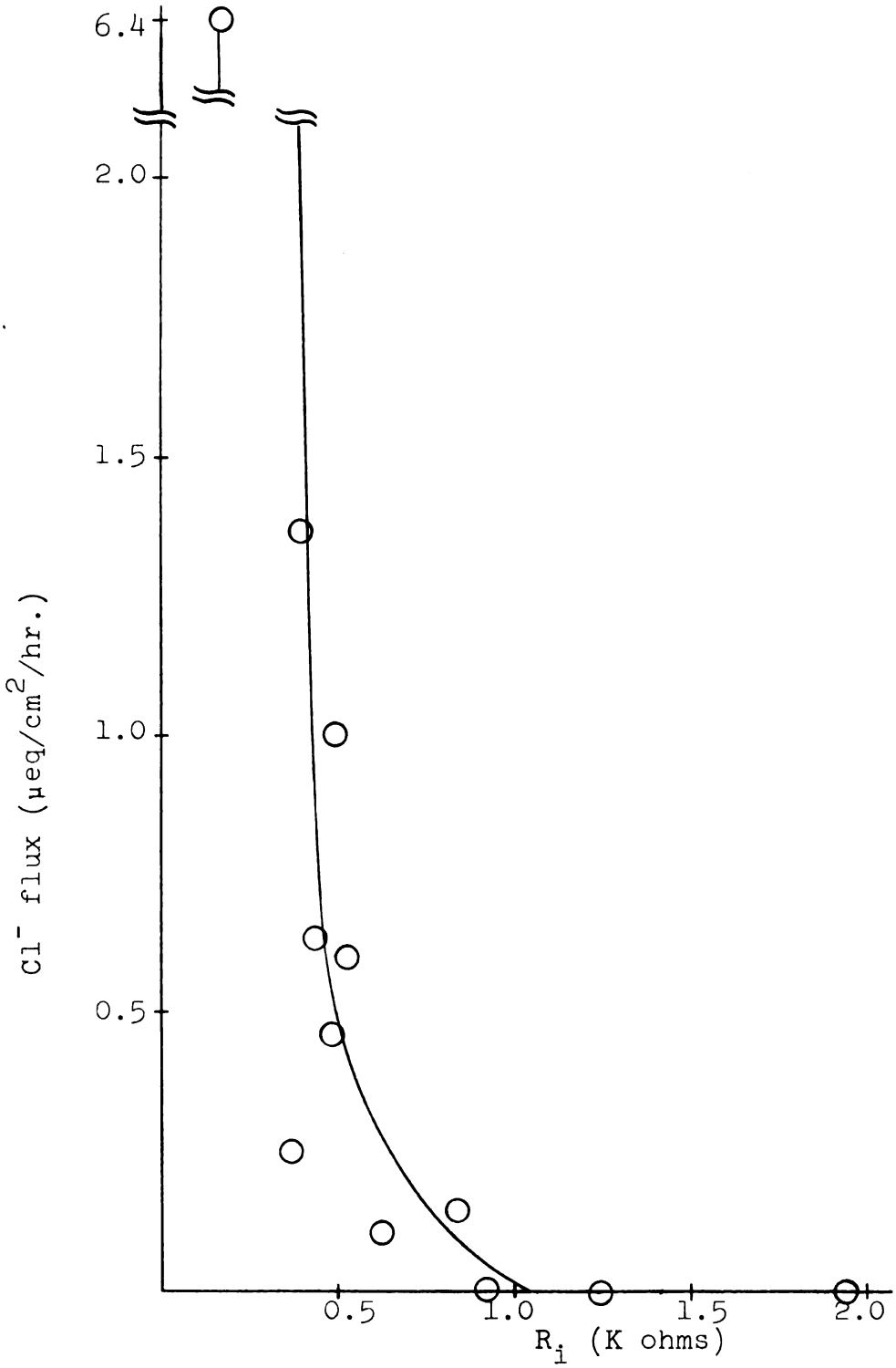


Figure 15.--Chloride flux versus R_i.

the R_i is 1000 ohms or larger the membrane has a low permeability to Cl^-; at lower R_i values the membrane permeability increases exponentially. A similar graph for Na^+ diffusion shows that the membrane is impermeable to the passive diffusion of Na^+ when the R_i is greater then 1500 ohms (Figure 16).

V. Prediction of the Crop Membrane Permeability to Water

The diffusion of water through the crop membrane can be predicted on the basis of the R_i. Water diffusion through the crop membrane as determined with HTO is greater when the R_i is small (Figure 17). At the highest membrane resistance reported in this thesis (7.0K ohm) the water flux was 2.0 meq/cm^2/hr.

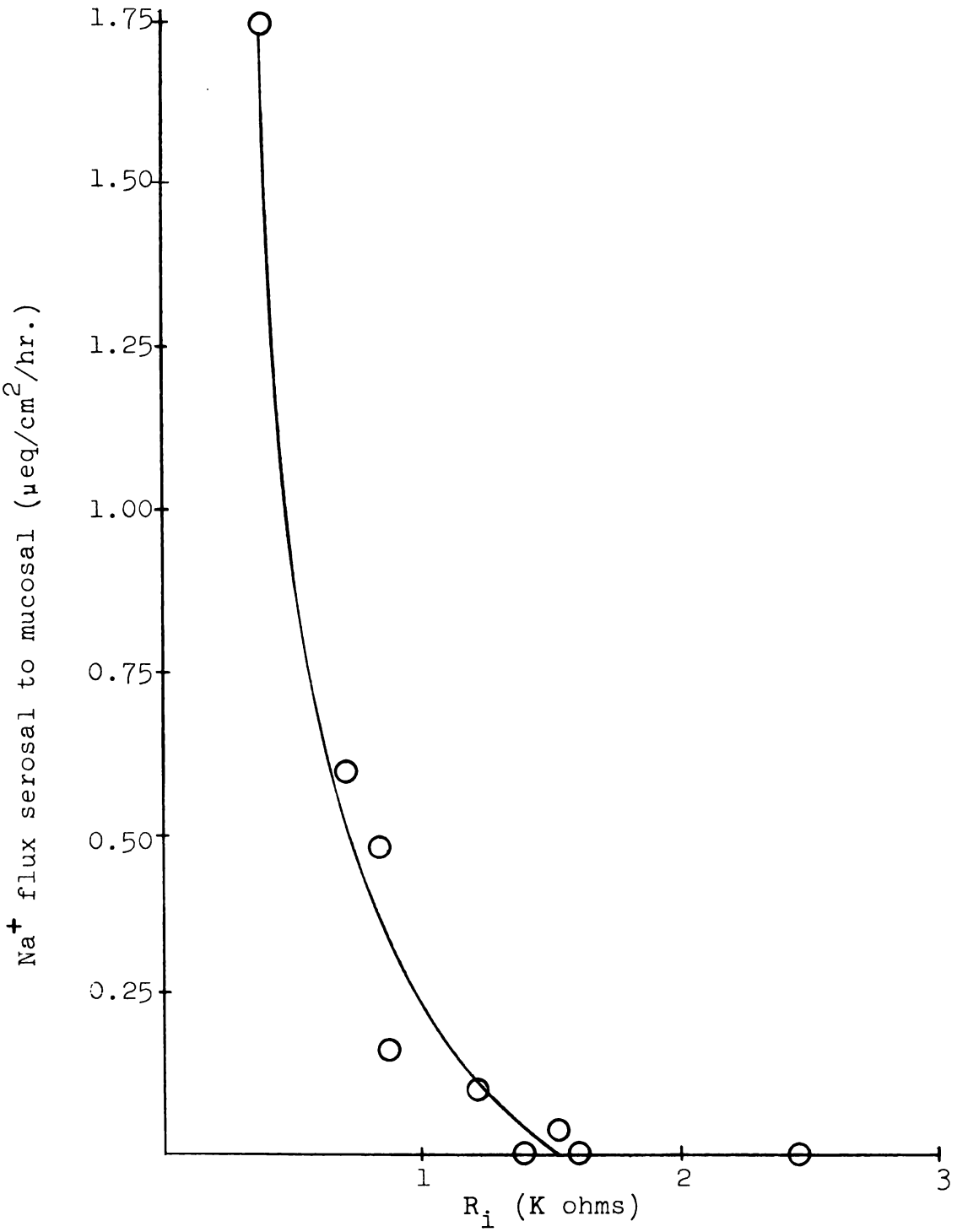


Figure 16.--Sodium flux serosal to mucosal versus R_i.

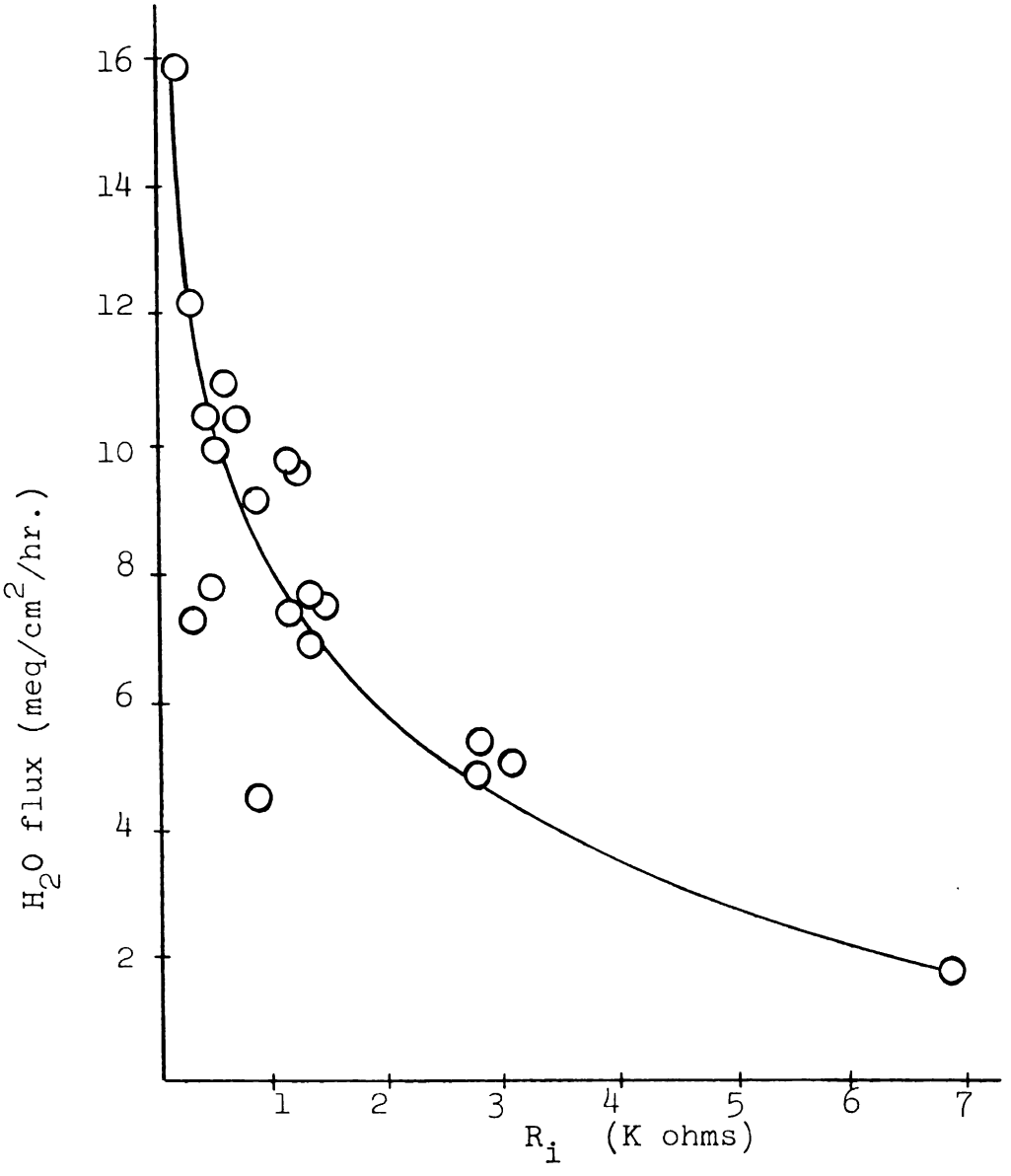


Figure 17.--Water flux versus R_i.

CHAPTER V

DISCUSSION

The first demonstration of active transport of ions through a biological membrane was made by Ussing (1951), working with frog skins. A. C. Brown (1965) summarized the conditions necessary for active transport as the following four points, (evidence is recalled under each point to support the author's hypothesis of active transport in the crop membrane):

(i) "the force is located within the membrane";

The force which moves Na^+ across the crop membrane must be located within the membrane since there is no concentration gradient between the perfusing solutions of the *in vitro* preparation.

(ii) "the force directly influences particle motion";

The force which accounts for the Na^+ accumulating in the serosal solution must be direct since under s.c.c. conditions there are no electrochemical or osmotic gradients influencing particle motion;

(iii) "the force tends to increase the free energy of the particle as it passes through the membrane";

The free energy of the Na^+ is greater in the serosal solution because they have a higher electrochemical energy

(positive p.d. under non-s.c.c. conditions) than the Na^+ in the mucosal solution.

(iv) "the force is established by and maintained through the consumption of free energy made available by metabolism";

The s.c.c. is dependent on the glucose available to the membrane, and it is inhibited by metabolic poisions.

Brown (1962) characterized the behavior of frog skins to ion diffusion by measuring what he referred to as "mean internal conductance" (σ = s.c.c./p.d.). He observed that σ usually falls for a short time immediately after the skin has been perfused in vitro. He hypothesized that σ become smaller because the p.d. is raised due, perhaps, to increased passive resistance.

Data from the present experiment support the idea that a change in passive ionic resistance can change the relation between the s.c.c. and the p.d. In this thesis, however, the above relation has been referred to as "internal ionic resistance," R_i = p.d./s.c.c. This term seems more applicable since the passive resistance to ions is being described; it has the additional qualities of being distinct and independent of the Na^+ pump activity.

To verify that the R_i describes the passive ionic resistance of the membrane independent of the s.c.c. the following experiment was done. A membrane was perfused in the usual manner for one hour during which the s.c.c. and

p.d. (and therefore the R_i) were constant. Ouabain was then put in the serosal solution to decrease the s.c.c. According to Schatzmann (p. 13) ouabain inhibits the Na^+ pump without altering the passive permeability of a membrane. The s.c.c. and p.d. were alternately measured as they decreased to zero. As seen in Table 4 the R_i did remain constant as the s.c.c. decreased and therefore is independent of the s.c.c.

TABLE 4.--Independence of R_i and short-circuit current.

Time (min.)	0	2	4	6	8	10	12	14	16	18
p.d. (mV)	22	21	17	15	14	12	9	8	6	5
s.c.c. (μA)	13	13	11	10	9	7	6	5	4	3.3
R_i (K ohms)	1.69	1.62	1.55	1.50	1.55	1.71	1.50	1.60	1.50	1.52

The concept of measuring the permeability of a biological membrane to ions on the basis of its electrical properties may be useful in evaluating the effects of other agents on membrane permeability. For instance, the activity of the Na^+ pump in the frog skin is known to be regulated by ADH (Koefoed-Johnsen and Ussing, 1953). Schwartz *et al.* (1960) have proposed that ADH stimulates the Na^+ pump activity of the frog skin by increasing mucosal permeability to ions which will cause increased amounts of Na^+ to be available to the Na^+ pump. It may be possible to evaluate Schwartz' theory of ADH action by observing the R_i as the hormone acts

on the skin. If ADH does increase the mucosal permeability of the frog skin a concurrent decrease in R_i would be expected.

In the in vitro crop membrane preparations ADH in low concentration (1 IU/ml serosal solution) has not increased the Na^+ pump activity. If the application of increased concentrations of ADH stimulates the Na^+ pump activity due to increased ion permeability of the epithelial cells, then this stimulation should be accompanied by a decrease in R_i.

It was reported (p. 39) that the crop membrane retains 89% less Na^{22}, 88% less Cl^{36} and 45% less HTO when the isotopes are introduced into the mucosal solution rather than the serosal solution. This different saturation ratio of Na^+, Cl^- and H_2O may be due to a functional permeation barrier in the membrane. The barrier in the crop membrane is assumed to be the germinativum as it is in the toad bladder (Edelman et al., 1963, and Leaf and Hays, 1962), the mucosa of the stomach (Rehm, 1963) and the intestine (Wilson, 1962) or the combined germinativum and spinosum as in the frog skin(Farquhar and Palade, 1966).

A cross section of the crop membrane (Figure 1) has a low layer of cornified epithelium (8μ) overlying the spinosum and germinativum (4 μ) and a thick submucosa (50 μ). Because the mucosa is one sixth the thickness of the submucosa it would be expected to have about 86% less Na^+ and Cl^- to exchange with the bathing medium than the submucosa has. The reported values for Na^{22} and Cl^{36} retention are

in agreement with the expected value assuming that the germinativum is the diffusion barrier.

The mucosa should also have 86% less H_2O to exchange with the bathing medium than the submucosa has. The results indicate, however, that only 45% less HTO is retained when this isotope is introduced into the mucosal solution than when it is introduced into the serosal solution. Evidently the permeability of the membrane to water is high enough that it becomes nearly saturated with HTO during the three to nine hour experiments. A series of short (10-60 minute) experiments should help determine whether HTO is more rapidly exchanged with the submucosa than the mucosa.

The literature reveals no successful methods of predicting H_2O diffusion rate through a biological membrane on the basis of electrical properties. Garby and Linderholm (1954) unsuccessfully attempted to correlate D_2O movement across the frog skin with electrical resistance used as an index of the permeability to ions.

The present experiments have indicated that H_2O diffusion through the crop membrane can be predicted on the basis of the R_i (Figure 17). There is no electrochemical or structural model which explains why the H_2O permeability is related to the R_i. However, from the similarity between Figures 15, 16, and 17 it is reasonable to assume some common factor determines the permeability of the membrane to both ions and H_2O.

CHAPTER VI

SUMMARY

An Ussing type perfusion system has been used to demonstrate that a sheet of tissue from a homeothermic animal (pigeon crop membrane) can be maintained in a viable state for up to twelve hours.

The crop membrane is electrogenic and may be compared with the frog skin and toad bladder as units which have ability to move Na^+ against an electrochemical gradient, i.e., this membrane has a "Na^+ pump." The pump activity has been shown to be decreased by DNP or by the lack of glucose. It is dependent on the presence of Na^+ in the mucosal solution and K^+ in the serosal solution.

The perfused crop membrane takes up Na^+, Cl^- and H_2O more rapidly from the serosal solution than from the mucosal solution. This has been explained in terms of a diffusion barrier located near the mucosal surface.

A method of predicting the permeability of a crop membrane to cations (Na^+) and anions (Cl^-) on the basis of the p.d./ s.c.c. has been presented. A membrane for which the p.d./ s.c.c. is greater than 1K ohm has a low permeability. It is expected that this method would be applicable to other membranes with electrogenic ion pump activity.

The permeability of a crop membrane to H_2O is also predictable on the basis of the p.d./s.c.c. Membranes for which this ratio is large have relatively low permeabilities.

The research described in this thesis has led to the following questions pertaining to the pigeon crop membrane:

1. What is the size of the Na^+ space and H_2O space and where is the permeability barrier located?
2. In what part of the membrane is the Na^+ pump ATPase located?
3. Is Li^+ or any other cation able to substitute for Na^+ in the pump mechanism?
4. How much oxygen is consumed in the transport of each Na^+ ion?
5. What is the effect of aldosterone and ADH on the Na^+ pump activity?
6. What effect does Ca^{++} have on the permeability of the crop membrane to ions and water?

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