FACTORS AFFECTING THE POTENTIAL, CURRENT AND RESISTANCE OF THE PIGEON CROP MEMBRANE IN VIVO AND IN VITRO

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RICHARD CARROL ROSE

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This is to certify that the

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Richard Carrol Rose

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Wm L. Trautman
Major professor

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ABSTRACT

FACTORS AFFECTING THE POTENTIAL, CURRENT AND RESISTANCE OF THE PIGEON CROP MEMBRANE *IN VIVO* AND *IN VITRO*

By

Richard Carrol Rose

The pigeon crop membrane *in vitro* has previously been shown to develop a transmembrane potential difference (PD) with the serosal surface positive as a result of an active transport of Na^+ in the direction mucosa to serosa. The present problem was to determine whether the crop *in vivo* and *in vitro* have the same electrogenic properties.

The Ussing technique has been used to study the crop *in vitro* and has been modified in the present experiments for use on the crop *in vivo*. When the membrane is bathed with a Ringer solution of approximately the same ionic composition as plasma the average initial electrical characteristics of 50 of the crop epithelial membranes *in vitro* were: PD = 22.7 mv; short-circuit current (Jsc) = 25.8 μA; R (= PD/Jsc) = 1.07 K ohms. Average initial values of 30 membranes *in vivo* were: PD = 24.7 mv; Jsc = 57.6 μA; R = 0.41 K ohms.

Ringer solutions bathing the mucosal (luminal) surface were depleted of Na^+ by either choline substitution or by water dilution. This depressed the Jsc both *in vivo* and *in vitro*. Only the water diluted (hyposmotic) solution caused a resistance increase *in vivo* (nine per cent) or *in vitro* (110 per cent). This resistance increase is explained as being due to imbibition of water by the tissue cells with a consequent restriction of extracellular paths available for passive ion diffusion.

Ringer solutions made hyperosmotic with sucrose, ethanol or dimethyl sulfoxide bathing the mucosal surface *in vivo* or *in vitro* resulted in a decrease in R and an increase in Jsc. The osmotic effect might again be due to alterations of extracellular pathways.

Substitution of $SO_4^=$ for Cl^- in the Ringer solution bathing the mucosal surface either *in vivo* or *in vitro* resulted in an increase in R. This is possibly because the membrane is less permeable to $SO_4^=$ and since this anion is unable to follow the actively transported Na^+ across the membrane a larger separation of charge develops.

The presence of Ca^{++} in the bathing solution reduced the Jsc and increased the R both *in vivo* and *in vitro*. It seems likely that the presence of Ca^{++} limits the passive movement of ions through some part of the membrane.

Restriction of Na^+ in the mucosal solution from the Na^+ transport cells would reduce the amount of Na^+ available in these cells and inhibit the transport process.

Cu^{++} in low concentrations bathing the mucosal surface both *in vivo* and *in vitro* resulted in an unexplained increase in the Jsc but no change in R.

A seasonal variation in the Na^+ transport rate has been noted but efforts to control the rate with aldosterone, pitressin, prolactin, epinephrine and lentin have not been successful.

Monovalent cations other than Na^+ at the mucosal surface and K^+ at the serosal surface of the crop are not effective in maintaining the membrane PD and Jsc.

Results of this study indicate that the crop *in vivo* is able to transport Na^+ from the mucosal to the serosal surface. The transport rate is great enough to assign tentatively to the crop a role in ion regulation of the whole animal. A model of this tissue is presented to explain its ion transport processes.

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By

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CHAPTER I

INTRODUCTION

Frantz and Rose (1968) have previously reported that the isolated pigeon crop membrane actively transports Na^+ in the direction mucosa to serosa. Many characteristics of the crop transport mechanism were found to be similar to those of anuran epithelia, e.g., Na^+ was required in the mucosal solution, K^+ was required in the serosal solution, the transport rate was decreased by 2-4 dinitrophenol (DNP), ouabain, a lack of nutrient supply or temperatures in excess of 41°C.

The short-circuiting technique of Ussing has been used extensively in crop membrane and other studies of *in vitro* ion transport because it allows the membranes to be examined under standardized conditions. The results of such *in vitro* studies coupled with other information have led to membrane transport models. However, only occasionally have these models been tested *in vivo*.

The transport characteristics of a membrane should be analyzed *in vivo* for three primary reasons: (1) if membrane transport models are to be complete they will have to describe the behavior of the membrane as it

interacts with its natural control systems; (2) a particular ion transport mechanism might be of significance to the animal for ion regulation. A quantitative analysis of the ability of a tissue *in vivo* to take up salt as compared with the total salt needs of the animal would give some indication of the importance of its transport process; (3) some characteristics of membranes may be altered in response to removing them from the animal or in response to the new environmental conditions when used *in vitro*. A model formulated only on the basis of information derived from *in vitro* studies might not be relevant to the whole animal.

The effects of Cl^-, Ca^{++}, Li^+, Rb^+, Cs^+ and H^+ in the Ringer solution on the potential difference (PD) and the short-circuit current (Jsc) of the crop *in vivo* and *in vitro* were evaluated. Pitressin, prolactin, epinephrine, aldosterone and lentin were administered in an attempt to determine whether these agents affect the rate of Na^+ transport by the crop.

CHAPTER II

REVIEW OF THE LITERATURE

Early Ion Transport Studies

In the early history of ion transport as reviewed by Kleinzeller and Kotyk (1960) DuBois-Reymond in 1848 was the first to observe that the frog skin maintains a potential difference between the inside and outside. Reid in 1892 postulated that the salt and water balance of some amphibians could be partially maintained by the absorption of NaCl by the skin. Galeotti in 1904 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 isolated frog skin and attributed this to the movement of salts by active transport (energy dependent transfer of a solute against an electrochemical gradient).

Huff (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 solutions. 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 μEq/hr/cm^2 from a solution of only 1 mM Cl^-. The rate of uptake increased as the external Cl^- concentration increased.

The simplicity of Krogh's study made the results rather difficult to interpret, however. For instance, it was not possible to determine whether Cl^- was actively transported inward or if it was merely diffusing down an electrical gradient created by the active transport inward of some cation. At this point in the history of ion transport study no technique had evolved which would allow one to distinguish between passive and active processes of ion movement, but the theoretical work of Nernst in 1890 helped to fill this void (Brown, 1965).

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

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

where:

E = potential difference across the membrane (mv)

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

F = Faraday's constant (96500 coulombs/Eq)

c_1 = ion concentration of inside solution.

T = temperature (degrees K)

z = valence

c_2 = ion concentration of outside solution

Ussing (1949) developed the following equation to describe the ratio of diffusion rates of an ion between two similar solutions having a maintained electrical potential difference, E':

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

where:

M_{in} = ion flux inward

M_{out} = ion flux outward

When E' across a membrane is zero, the ratio M_{in}/M_{out} for any specific ion which is equally distributed across the membrane should be 1; i.e., there should be no net flux of the ion if it diffuses freely. However, if an electrical potential is maintained across the membrane the ion diffusion will be influenced. At 25°C a 58 mv potential, for example, will result in a ratio M_{in}/M_{out} of 10. The unidirectional fluxes can be

conveniently estimated by using two different radioisotopes of the same ion, as ^{22}Na and ^{24}Na.

Ionic behavior which deviates from the Ussing equation is not conclusive evidence, however, that the ion is actively transported rather than purely passively distributed. For instance, Anderson and Ussing (1957) have shown that solvent drag can influence the movement of an ion. The technique of Ussing and Zerahn (1951), referred to as Ussing's short-circuit technique, finally allowed one to determine if a particular ion moved by a process of active transport.

Ussing's Short-Circuit Technique

The apparatus used by Ussing (Figure 1) served the same purposes as that of the present experiment. The skin, S, was placed between two chambers, C, which held Ringer solution. Two narrow agar Ringer bridges, A and A', made contact with the Ringer solution very close to the skin. The outer ends of A and A' made contact with saturated KCl-calomel electrodes. The potential difference across the skin was read on a tube potentiometer, P. Another pair of agar-Ringer bridges, B and B', opened at the ends of the chambers. The outer ends of these bridges led to beakers with saturated KCl saturated with AgCl. Silver wire immersed in these beakers served as electrodes through which an external electromotive force (E.M.F.) was applied. The voltage was supplied

from the dry cell battery, D, and adjusted with the potential divider, W, so that the potential difference across the skin was maintained at zero. The current used in this process was read on the microammeter, M, and referred to as the short-circuit current (Jsc).

The unidirectional flux of Na^+ across the membrane was measured in one direction using ^{22}Na and simultaneously in the other direction using ^{24}Na. The calculated net flux of Na^+ was found to be very close to the amount of current (number of electrons) supplied by the external circuit. Ussing thus considered the Jsc to be a direct measure of the Na^+ transport rate under these conditions. The Ussing technique has subsequently been applied to many other biological membranes in vitro.

Models of Na^+ Transport

Koefoed-Johnsen and Ussing (1958) presented a model (Figure 2) illustrating their hypothesis about the origin of the frog skin potential on the basis of their studies in which various concentrations of Na^+ and K^+ bathed the inside and outside surfaces of the skin. Intracellular Na^+ concentration is low and extracellular Na^+ concentration is high, while intracellular K^+ is high and extracellular K^+ is low. They postulated that Na^+ diffuses into the cell from the outside solution. Na^+ is then actively transported across the inner border, perhaps in exchange for K^+ from the inside solution.

Figure 1.--Diagram of Ussing's short-circuiting apparatus. (Ussing and Zerahn, 1951).

C = chamber for containing Ringer solution

S = skin

a = inlet for air for stirring

A and A' = agar-Ringer bridges for connecting solutions with calomel electrodes

B and B' = agar-Ringer bridges for applying outside E.M.F.

D = battery

W = potential divider

M = microammeter

P = tube potentiometer

Figure 2.--Ion pumping system of the epithelial cell. The ion pumping system of the anuran epithelial cell as described by Koefoed-Johnsen and Ussing (1958) is illustrated. The outward-facing cell membrane (O.c.m.) is permeable to Na^+ and the inward-facing cell membrane (I.c.m.) is permeable to K^+. The pump (P) actively transports Na^+ from the cell and K^+ into the cell.

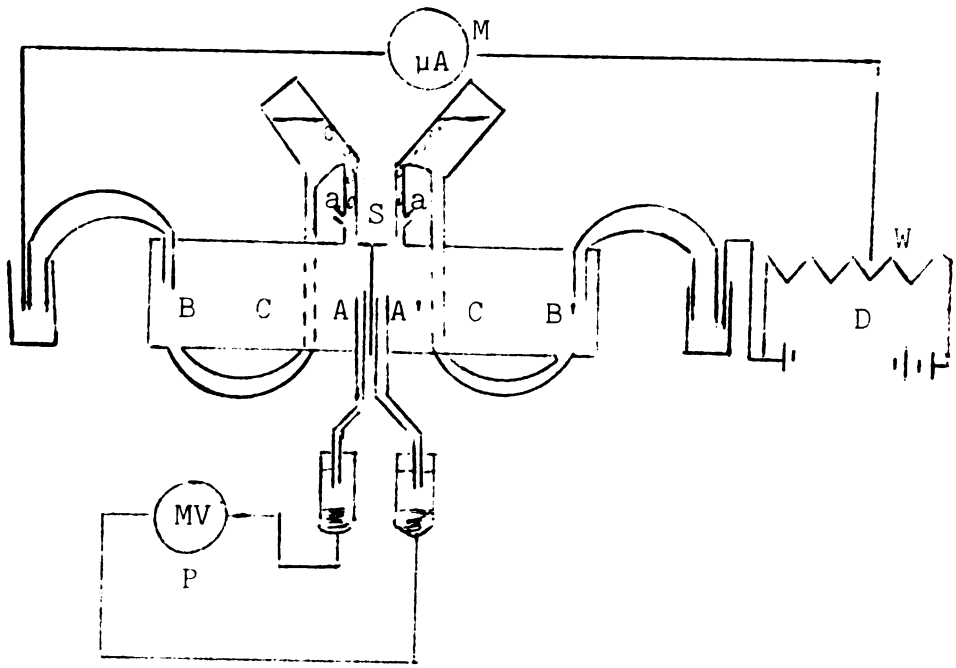

Figure 1

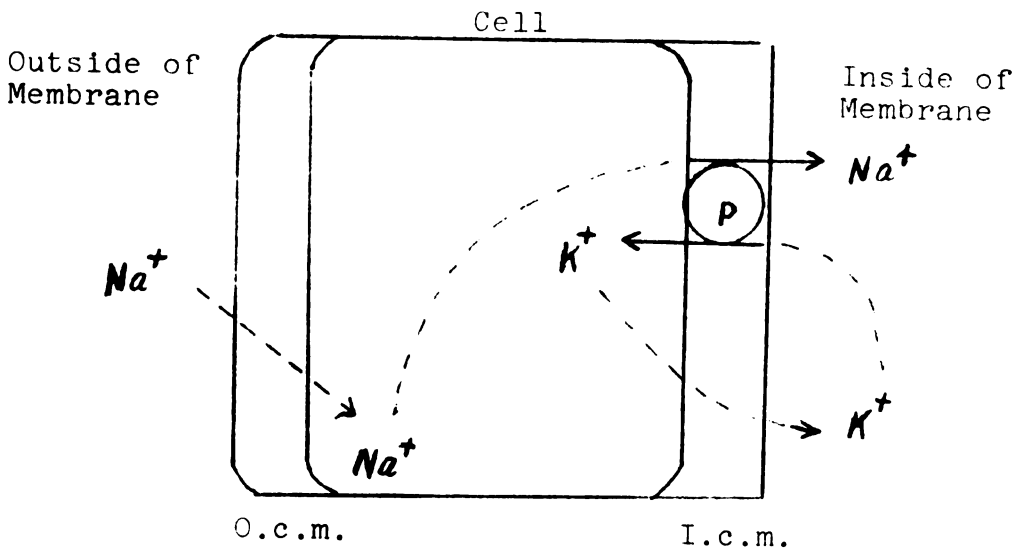

Figure 2

The K^+ which is pumped into the intracellular spaces diffuses down its concentration gradient back into the inside bathing solution, which makes the inside solution positive in relation to the cell. From anatomical evidence, the sodium selective (Na^+ permeable, K^+ impermeable) membrane was assumed to be the outward-facing membrane of the stratum germinativum.

Farquhar and Palade (1966) have used photomicrographs in their study of the localization of ATPase activity in the epidermis of frogs and toads in an attempt to develop a more accurate model of ion transport than Koefoed-Johnsen and Ussing presented. Since ATPase was shown by Bonting and Caravaggio (1963) to participate in the ion transport process, Farquhar and Palade used the presence of membrane ATPase as a marker of the transport site. The enzyme was found to be present in all cell membranes that face the labyrinth of epidermal extracellular spaces. No activity was indicated in the outer and inner fronts of the epidermis or where the surfaces of two cells come in contact.

From their results Farquhar and Palade suggest modifications on the Koefoed-Johnsen and Ussing model of the frog skin (Figure 3). The recently proposed model allows the membrane a larger surface area for pumping activity. Farquhar and Palade suggest that the sodium-selective membrane should be located nearer the

outside border of the epidermis since there appears to be no structurally continuous barrier on the outer side of the s. germinativum as Koefoed-Johnsen and Ussing assumed. Farquhar and Palade proposed 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 PD across the frog membrane can be used 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 most skins 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 50 microns. The full potential (73-145 mv) of each membrane was recorded when the micro-electrode reached a depth of about 100 microns.

The results of Engback and Hoshiko appear to be more consistent with the theory of Farquhar and Palade than with the interpretation of Koefoed-Johnsen and Ussing. The two step development of the PD may be due

Figure 3.--Model of amphibian epidermis. This figure depicts the 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 stippled gray. EM, external medium; OFM, outward-facing membrane; IFM, inward-facing membrane; BM, basement membrane; IM, internal medium; d, desmosome.

Figure 4.--Carrier based transport model of erythrocyte as diagrammed by Shaw (1955). X and Y represent two forms of the carrier molecule which combine with K^+ and Na^+ to form uncharged complexes which can cross the membrane. The conversion of form X to form Y at the inside surface requires energy.

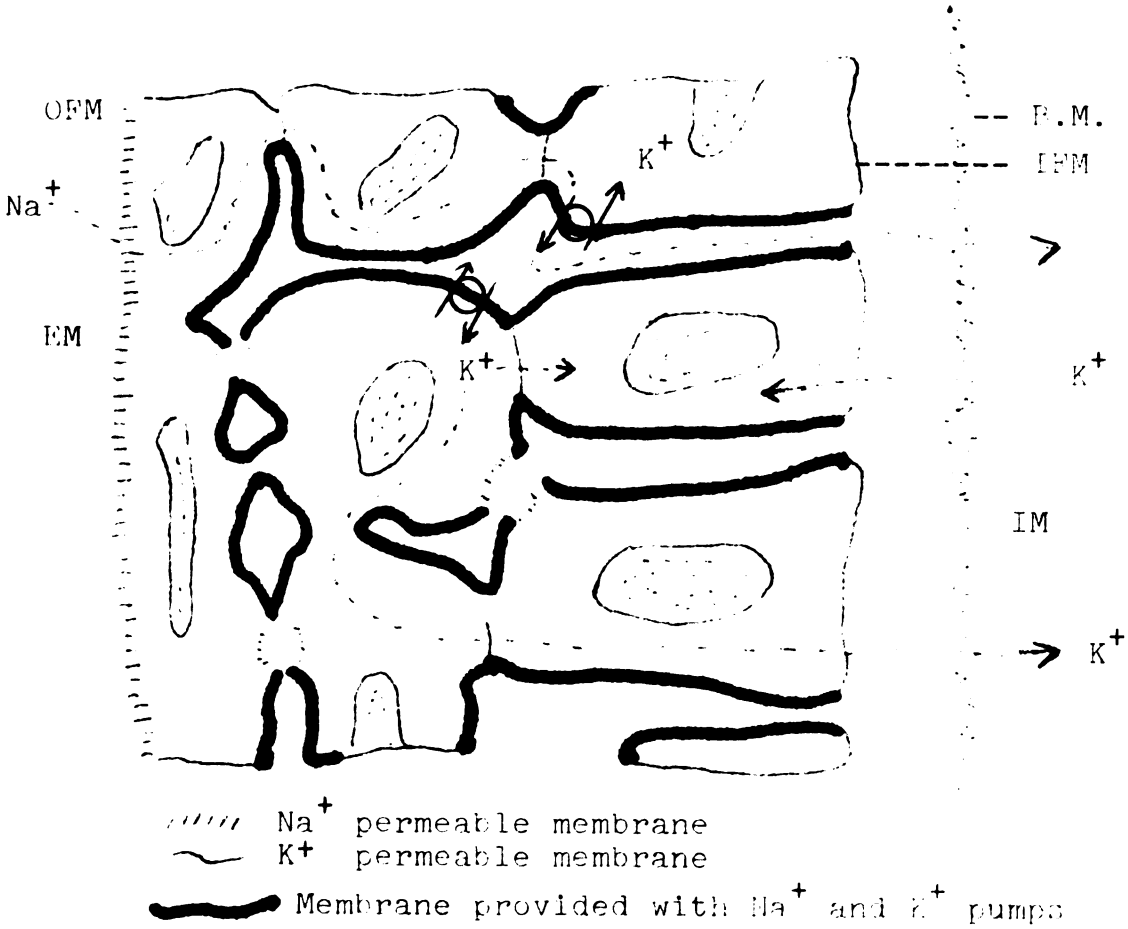

Figure 3

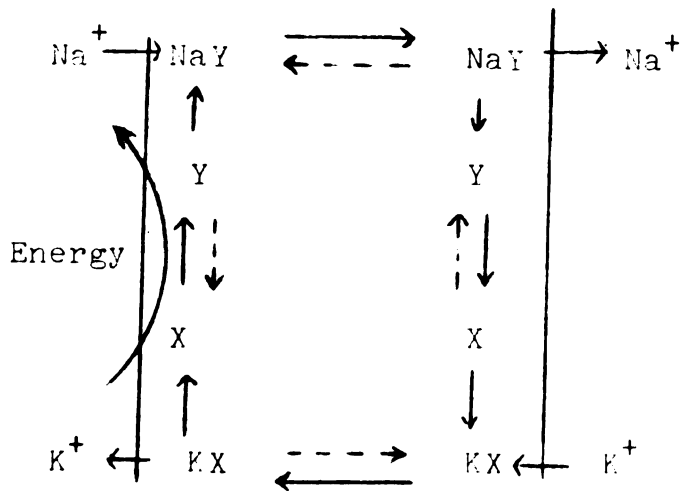

Figure 4

to the diffuse distribution of the Na^+ pumps as shown in Figure 3.

Mechanism of Sodium Transport

Models have been developed to describe how the ion transport mechanism of a cell membrane functions. The model described in Figure 4 was presented by Shaw (1955) to show how Na^+ and K^+ can be actively transported across the erythrocyte membrane. Intracellular Na^+ diffuses into the membrane and combines with the carrier molecule Y to form an uncharged complex which brings Na^+ to the outside surface. After releasing Na^+ at the outside surface, the membrane carrier changes from form Y to form X. Form X is able to cross the membrane only after it has associated with K^+ which is released at the inside surface. Energy (ATP) is required to convert the carrier from form X to form Y before the cycle can be repeated.

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 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.

Hormonal Action on Membranes

The neurohypophyseal hormone, ADH, has been found to alter the rate of movement of salts and water across many biological tissues. Fuhrman and Ussing (1951) found that ADH increases the Na^+ pump activity of the frog skin, and Ussing (1955) found that the permeability of the skin was increased. According to Leaf (1960) ADH increases the membrane permeability to water, and Maffly, Hays, Lamdin and Leaf (1960) found that ADH increases the membrane permeability to urea, cyanamide, aceteamide, propionamide, butyramide, dimethylformamide and nicotinamide.

At least two different theories are currently defended concerning the action of ADH on the permeability of membranes. Schwartz et al. (1960) and Anderson and Ussing (1957) 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) suggested 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. Edelman, Bogoroch and Porter (1963) demonstrated that in the toad bladder aldosterone activity is located in the nucleus of the epithelial cells and acts by promoting DNA-dependent RNA synthesis that produces proteins involved in the coupling of metabolism with Na^+ transport.

Non-Penetrating Anion Studies

Ussing (1951) recognized that the potential across a particular frog skin might be in part regulated by the ease of diffusion of anions (most frequently Cl^-) down the electrical gradient from outside to inside. Such diffusion would tend to "short out" the PD developed by the membrane. Due to the slow penetration of $SO_4^=$, its replacement for Cl^- in the outside bathing medium causes the isolated frog skin to develop a larger PD even though the Na^+ transport rate (and, therefore, the Jsc) remains unchanged. Because of the larger potentials, much work is done on anuran skins with the use of Cl^- free Ringer solutions.

Ion Replacement Studies

The ability of a biological membrane to recognize the various monovalent ions and treat them differently is not precise. When Zerahn (1955) replaced part of the Na^+ of the outside solution of frog skin by Li^+, the Jsc was larger than the net Na^+ transport, indicating that the new ionic species was able to support some current. When Na^+ was partially replaced by K^+ the Jsc was reduced but was still equal to the net transport of Na^+. These results were interpreted by Zerahn to indicate that Li^+ could partially replace Na^+ in the transport mechanism but K^+ could not.

The Na^+ pump mechanism of frog skin appears to be dependent on the presence of K^+ in the inside solution. Fukuda (1955) found that if a K^+ free solution was used as the inside solution, the potential difference was reduced very rapidly to zero. Harris and Maizels (1951) showed that the active transport of Na^+ was reduced under these circumstances. Rb^+ was found to be able to replace K^+ almost completely and Cs^+ replaced it partially. The ability of cations to substitute for K^+ at the inside surface of bullfrog and leopard skins has been studied by Lindley and Hoshiko (1964). They reported that the order of selectivity of replacement is Rb > Cs > Li > Na.

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Teorell (1954) has reported that Li^+ bathing the outside surface of frog skin causes rhythmical oscillations of the impedance and potential. Li^+ (20-300 mM) produced sinusoidal variations of the PD of 0.1 to 10 mv and a frequency of 0.3 to 1.0 per minute. Other workers using similar preparations have not been able to demonstrate this interesting phenomenon.

Hyperosmotic Solutions, Hyposmotic Solutions

T. C. Barnes (1939) reported that D_2O Ringer bathing the inside and outside of isolated frog skin caused a decrease in the membrane potential. S. C. Brooks (1937) suggested that the D_2O Ringer had a hyperosmotic effect while bathing the skin. Lindley, Hoshiko and Leb (1964) evaluated the effect on frog skin of Ringer solutions made hyperosmotic with D_2O, sucrose, mannitol, acetamide, urea, thiourea, Na_2SO_4 and K_2SO_4. Skin potential and R decreased when the outside solution was made hyperosmotic by these agents. When the outside solution was made hyposmotic, the R increased or remained unchanged. Hoshiko could not explain why some solutes had a greater effect on R than others.

A. C. Brown (1962) experimented with hyposmotic Ringer solutions bathing the frog skin *in vitro* and *in vivo*. His data show that the resistance under both conditions was increased in response to the hyposmotic

Ringer. Brown explained the higher resistance as being due to a lower Cl^- concentration in the diluted solution. He reasoned that the charge separation across the membrane would be greater when Cl^- was not present to follow the actively transported Na^+. This would result in an increase in the calculated R.

Ethanol Effect on Active Transport

Israel and Kalant (1963) have used the frog skin in an attempt to elucidate the effect ethanol has on the cellular mechanism of active ion transport. This effect would be of particular interest if it represents the action of alcohol on the function of nerve tissues. Ethanol in "non-lethal" (54-217 mM) doses was used in the Ringer solution bathing the outside of the skin. At the highest dose there was a 50 per cent reduction of the Jsc within 90 seconds. Alcohol in the inside solution had variable effects on the Jsc.

Metabolism and Transport Inhibitors

Francis and Gatty (1938) suggested that NaCl movement through the frog skin was dependent on metabolism since 1 mM cyanide, a known metabolic inhibitor, abolished the net flux. Bromo-acetate reduced the net inward Cl^- movement which was restored with the addition of pyruvate or lactate. Schoffeniels (1955) found that 2-4 dinitrophenol (DNP) caused the frog skin potential to

fall and finally to reverse. In the inactivated membrane, Na^+ movement was passive and with Ringer solution at each surface there was no net flux. Frantz and Rose (1968) reported that the Jsc of the isolated pigeon crop was reduced by the presence of DNP in the serosal bathing solution, indicating that the Jsc is dependent on a metabolic supply.

Zerahn (1956) and Leaf and Renshaw (1957) compared oxygen consumption rates with the active transport of Na^+ in the frog skin. Zerahn compared O_2 consumption of the normal perfused membrane with that of a skin in a Na^+ free solution (inhibited transport activity). Leaf and Renshaw 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. When Leaf and Renshaw placed the skin in an oxygen-free Ringer solution the Na^+ transport decreased to 10 per cent of its original value within 30 minutes, showing that anaerobic metabolism is not sufficient to support the transport process.

Koefoed-Johnsen (1957) reported that the presence of ouabain reduces the frog skin potential difference. This may be due to its inhibitory effect on ATPase activity. Schatzmann (1953) suggested that ouabain has an inhibitory action at the site for K^+ activation of ATPase 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. Frantz and Rose (1968) reported that ouabain in the serosal solution resulted in a reduction of the Jsc of the pigeon crop *in vitro* indicating that the transport mechanism of this tissue also depends on ATPase activity.

Estimating Passive Ion Fluxes

Rose and Frantz (1967) reported an inverse correlation between transmembrane passive fluxes of Na^+, Cl^- and water and the calculated resistance of the individual membranes. Rose (1967) referred to the ratio of PD/Jsc as "internal ionic resistance" rather than d.c. resistance since the former term focuses one's attention on Na^+ and Cl^- permeabilities, rather than on electrical resistance. Experiments done on isolated newt skin by Gieske (private communication) indicate that transmembrane passive fluxes of Na^+ and Cl^- correlate inversely with R values.

From these observations Rose (1967) has devised a technique of estimating the passive unidirectional ion fluxes across a given membrane by measuring the PD and Jsc, calculating the resistance and referring to a standard curve as shown in Figure 5. In the case of the crop membrane, both Na^+ and Cl^- fluxes were below 0.05 $\mu Eq/cm^2/hr$ if the membrane R exceeded 1.5 K ohms. In spite of the steep slope in the R range, 0.8 to 1.5 K ohms for this particular tissue, the method has served

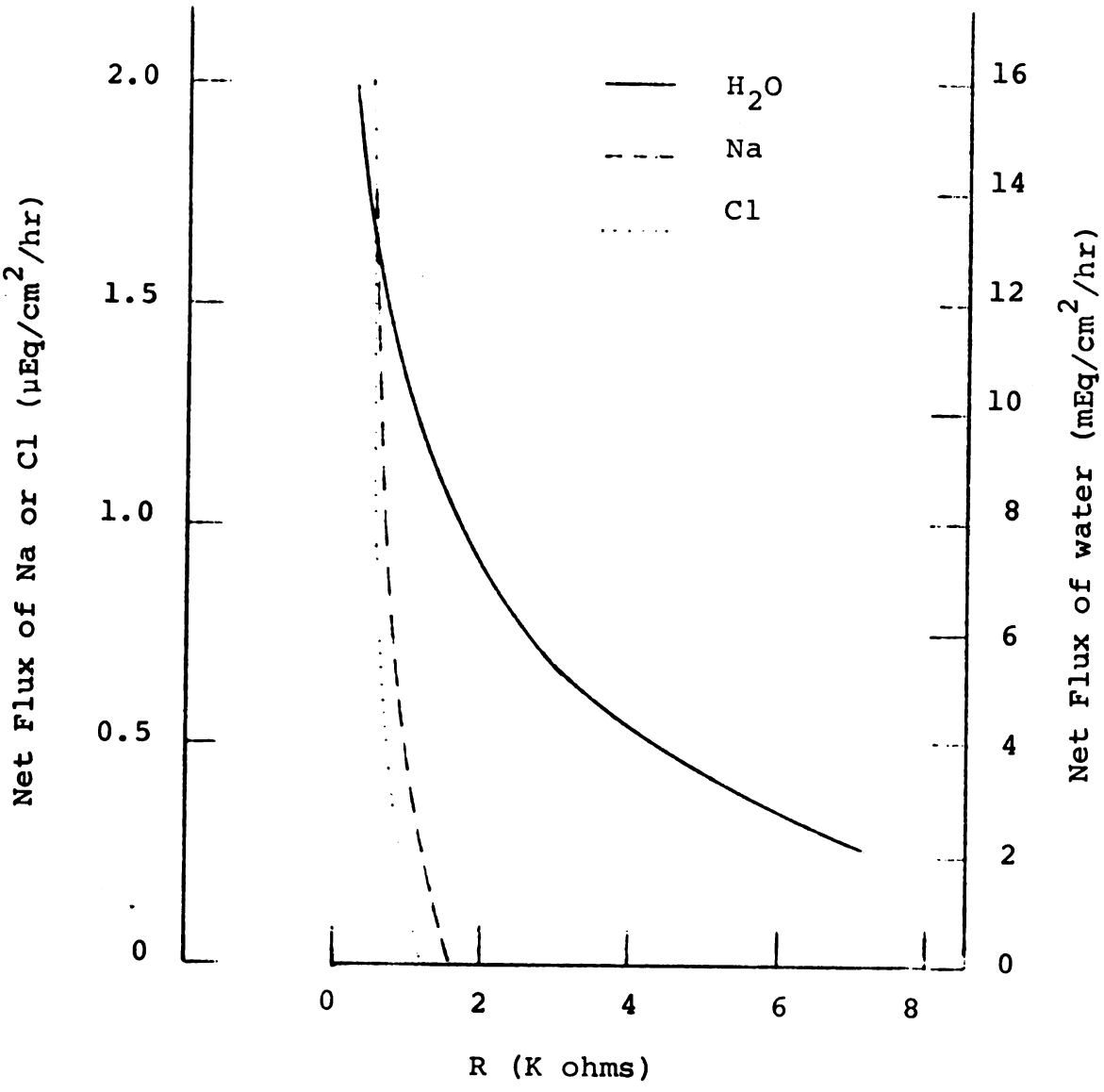

Figure 5.--Passive fluxes of Na^+, Cl^- and H_2O vs. R. of <u>in vitro</u> crop membranes. Rosé (1967).

the author well in the absence of an alternate method for quick identification of crop tissues which have very low permeability characteristics.

Effect of Ca^{++} on Permeability

Herrera and Curran (1963) found that Ca^{++} (11.3 mM) in the outside bathing solution of isolated frog skin decreased the Na^{+} transport and the Cl^{-} influx. Curran, Herrera and Flanigan (1963) found that the primary effect of Ca^{++} is on the passive Na^{+} permeability of the outward-facing membrane of the cells. They suggested that the rate of active Na^{+} transport is altered because the Na^{+} pool size in the transport cells is decreased.

Anatomy of the Pigeon Crop Membrane

Dumont (1965) has used light and electron microscopy in a study of the bilobed pigeon crop membrane. He reports the luminal (mucosal) surface of the crop membrane has a stratified squamous epithelium approximately twelve cells thick. The lamina propria is located beneath the epithelium 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.

The epithelium of the crop has been described in terms of two layers on a functional basis. Beams and Meyer (1931) called the superficial layer lining the lumen of the crop the "nutritive layer." This stratum

is 8-10 cells thick in the non-brooding bird and becomes 2-3 times thicker due to the production of more cells when the young hatch. The deeper or basal cells of the epithelium were referred to by Litwer (1926) as the "proliferating epithelium." It is here that the new cells are formed which are gradually pushed toward the lumen.

CHAPTER III

METHODS

Experimental Animals

White King pigeons (Cascade Squab Farm, Grand Rapids, Michigan) used in this study were 2-6 years old and of either sex. Fresh tap water and commercial feed (Allied Mills, Chicago, Illinois) were provided daily. The birds were kept in a room which received light only through unshaded windows. The temperature of the room fluctuated with the ambient temperature from 35°F up to a maximum of approximately 80°F at which temperature an air conditioner turned on to maintain the maximum temperature. None of the birds was observed to be producing "crop milk."

In Vitro Studies

Crop membranes used for *in vitro* experiments were quickly removed from birds killed by cervical dislocation. One tissue sample was taken from the ventrolateral area of each lobe of the crop sac. Each piece of tissue was held as a flat sheet between a pair of interlocking lucite rings. The tissue was placed in warm (37°C) Ringer solution and the serosa, muscle layers and most

of the submucosa were stripped off under a dissecting microscope. The mucosa and remaining submucosa were held between two lucite chambers exposing 2.54 cm^2 of membrane to 4 ml of stirred, warm (37°C) Ringer solution on each side (Appendix II). This preparation took 10-15 minutes.

The balanced salt solution (Ringer solution) used had the following composition (except in cases noted): Na^+, 145; K^+, 3.25; Cl^-, 128; Mg^{++}, 2.0; $SO_4^=$, 2.0; Ca^{++}, < 0.3 mEq/liter; glucose, 5.55 mmoles/liter; and buffered with tris(Hydroxymethylaminomethane) 10.0 mEq/liter. There has not previously been an extensive use of the crop membrane in vitro and therefore no pigeon Ringer solution appropriate for such studies was available. The formulation above was the result of modifying (lowering the concentrations of Ca^{++} and K^+ and eliminating the addition of HCO_3^-) the bird Ringer solution described by Prosser (1961). The concentrations of Mg^{++} and Ca^{++} were measured with an atomic absorption spectrometer and Na^+ and K^+ were measured with a flame photometer at the Soil Science Laboratory, Michigan State University. The osmolarity of the solution, measured by the method of freezing point depression with a Fiske Osmometer was 285 mOsM. The pH (measured on a Beckman Zeromatic pH Meter, Beckman Instruments, Inc., Fullerton, California) was adjusted with HCl to 7.6.

Miniature calomel electrodes (S-30080-17, E. H. Sargent and Co., Detroit, Michigan) were used in the circuit for measuring transmembrane electrical potentials. Their saturated electrolytic solution was changed from KCl of the manufacturer's specifications to NaCl because the K^+ released by the electrodes was found to alter the membrane potentials after 2-3 hours. Electrode pairs were checked before and after each use and confirmed to have an asymmetry potential of less than 0.5 mv.

Short-circuiting electrodes were prepared using silver wire, a 1.5 volt battery source and Ringer solution. Approximately 2 inches of silver wire was attached to each terminal of the battery and loose ends were inserted into a beaker of Ringer solution. After a thin coat of gray silver chloride was visible on the wires they were inserted into 5% agar-Ringer (5 g agar dissolved in 100 ml Ringer solution) plugs of 5 mm inside diameter which connected with the distal ends of the lucite chambers (see Appendix II).

The Ussing technique described in the review of the literature has been improved by the development of equipment by Ussing and Windhager (1964) which automatically nulls the transmembrane PD to zero and records the amount of current necessary in this process. The present experiment employed a Sargent model SR recorder

converted to a cathode follower for this purpose (see Appendix II). The Jsc was read directly off the chart as 1 μA/scale unit (0.1 inch). This external circuit was periodically broken, allowing the spontaneous membrane PD to develop and be measured using the Sargent recorder as a standard potentiometer.

In Vivo Studies

Pigeons were prepared for the *in vivo* measurement of potentials and short-circuit currents by anesthetization with 15 mg/Kg Na pentobarbital injected intramuscularly (im). The level of anesthesia was maintained by injecting additional Na pentobarbital (0.5 mg) whenever the corneal reflex returned (about once per hour). The level of anesthesia did not correlate with the electrical measurements. Na pentobarbital in the serosal bathing solution (15 mg/liter) *in vitro* was found to have no measurable effect on the electrical activity of the membrane.

Feathers covering the skin over the crop were removed and the bird was taped in a supine position (Figure 6) to an inclined board. A 3 cm longitudinal incision was made through the ventral skin of the neck; the esophagus was transected and the distal end extended out of the neck. The trachea was transsected and the distal end cuaterized and exposed to the air. Fluid which accumulated in the trachea was periodically

Figure 6.--Schematic view of preparation for the *in vivo* measurement of crop PD and Jsc. Anesthetized pigeon has PD sensing and short-circuiting electrodes for serosal solution seated in agar plug of 2.54 cm^2 cross section. Electrodes for mucosal solution are inserted via esophagus into crop lumen.

A. Inclined board.
B. Crop wall.
C. Rubber plug in caudal end of crop.
D. Ringer solution in crop.
E. Trachea.
F. Esophagus.
G. Suture connected to skin.
H. Skin chamber.
I. Agar plug, 2.54 cm^2.
J. Potential sensing electrode at serosal surface.
K. Short-circuiting electrode at serosal surface.
L. Short-circuiting electrode of mucosal solution.
M. Potential sensing electrode of mucosal solution.

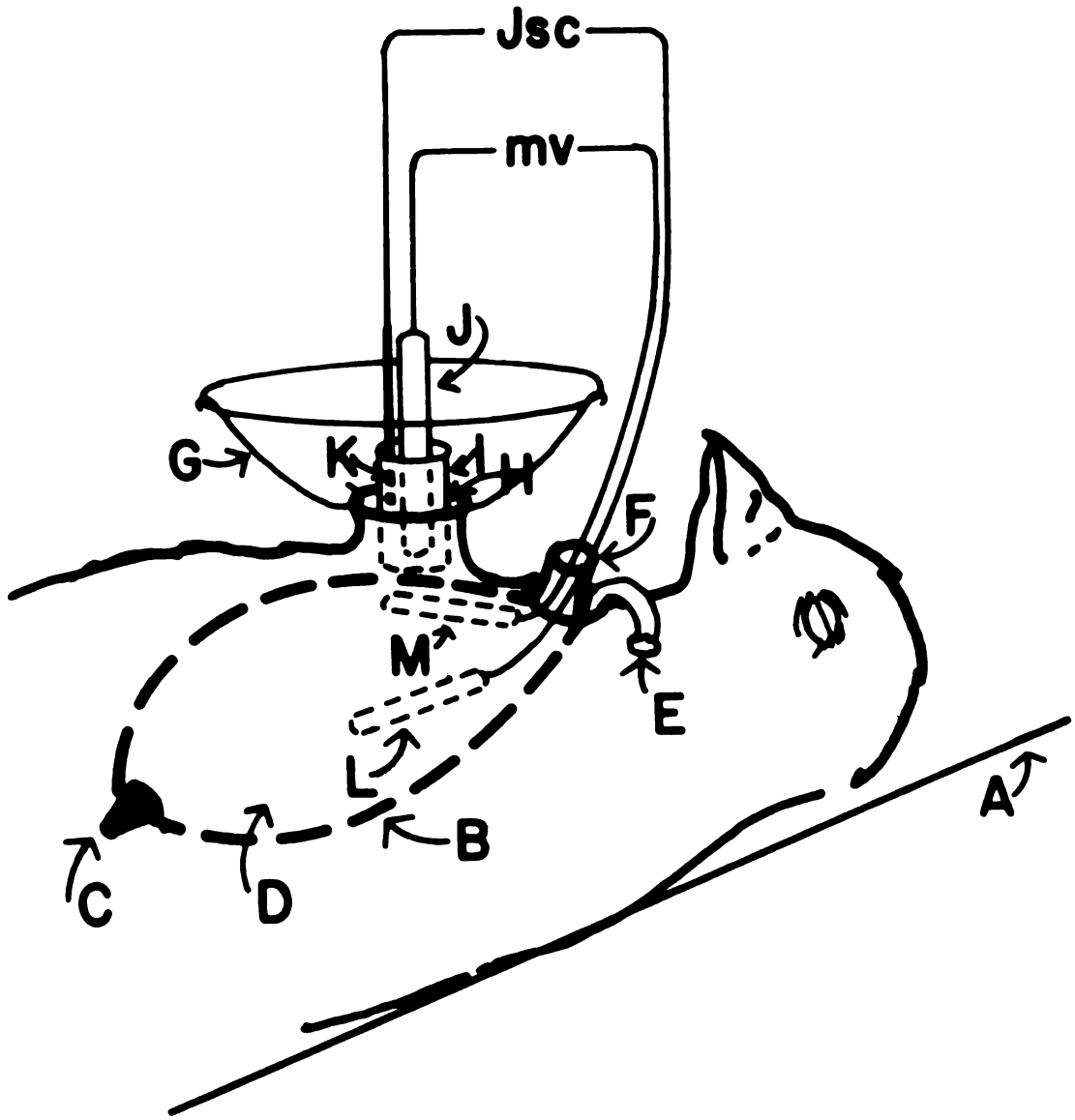

Figure 6

removed with a pipe cleaner. The lumen of the crop was washed several times with warm Ringer solution and inspected to insure that all food had been removed. The caudal end of the crop was sealed off with a soft rubber plug inserted using a hemostat via the esophagus to prevent loss of fluid through the rest of the digestive tract.

Electrodes used for in vivo studies were the same type as those used in the in vitro studies. The potential sensing electrode for the mucosal bathing solution was inserted via the esophagus into the crop lumen and the tip was secured a few mm from the mucosal surface of the crop. The short-circuiting electrode for the mucosal solution was inserted via the esophagus and the tip secured about 3 cm below the potential electrode. The position of all electrodes was maintained by clamps (not shown in Figure 6) located adjacent to the body of the bird. A polyethylene tube (P.E. 190) inserted via the esophagus was used to fill and empty the crop lumen of Ringer solution.

An incision was made through the skin (but not into the crop tissue) over one lobe of the crop sac marking the center of the area where electrical measurements would be made. The skin was pulled with sutures away from this point in all directions to expose the crop wall and to make a small chamber with skin forming the walls of the chamber.

Two different methods were used for arranging the electrode pair at the serosal surface. In early experiments a small pool (about 1 ml) of Ringer solution was held on the serosal surface of the crop by the skin chamber. The potential sensing and short-circuiting electrodes for the serosal solution were inserted directly into this pool of Ringer solution. The disadvantage of this technique is that the volume of Ringer solution is decreased either by evaporation or by absorption into the skin and crop membrane. Stable values of Jsc and PD were measured only if the pool of Ringer solution was frequently replaced.

The second method of locating the pair of electrodes at the serosal surface made use of a technique, Watlington, Campbell and Huf (1964) developed for measuring the PD and Jsc of frog skin in vivo. A 5% agar-Ringer plug of 2.54 cm^2 cross-section and 4 cm in depth was placed on the surface of the exposed crop and the tip of the potential sensing electrode was seated in the plug with the tip approximately 3 mm from the crop surface. The short-circuiting electrode was inserted into the agar plug with the tip about 3 cm from the crop surface. Friction tape was wrapped around the circumference of the agar plug to shield it electrically from the skin and to minimize evaporation of water from the plug.

Maintenance of blood circulation within the crop was demonstrated in several pilot experiments with the use of ^{22}Na injected intravascularly in a wing vein. After various periods of time (30 sec. to 3 min.) the agar plug was removed and the end which had been resting on the crop was analyzed for ^{22}Na using a liquid scintillation spectrometer (see Appendix III). ^{22}Na was detectable in the plug only 60 sec. after the isotope injection, indicating that blood circulation was not seriously retarded.

Grounded copper wire screen surrounded the *in vivo* and *in vitro* experimental apparatus to prevent spurious magnetic or electrical impulses from affecting the PD and Jsc measurements.

Most tests of experimental procedures or solutions followed a control period on the same animal and the paired Student t test was used for statistical analysis. In some experiments (as noted in Results section) one group of animals served as controls for another group of treated animals.

CHAPTER IV

RESULTS

Electrical Activity of the Crop Membrane In Vitro and In Vivo

The crop develops a transmembrane potential (serosa positive) when examined either *in vivo* or *in vitro* (Table 1). The electrogenic property of a biological tissue can be expressed in terms of the Ohms law relation E = IR, where E corresponds to the transmembrane PD, I corresponds to the short-circuit current (Jsc) and the membrane resistance, R, can be calculated as PD/Jsc.

TABLE 1.--Average electrical activities of crop membranes *in vivo* and *in vitro*.

	Jsc (μA)	PD (mv)	R (K ohms)	N
In Vivo	57.6 ± 5.4	24.7 ± 3.3	0.41 ± 0.11	30
In Vitro (scraped)	25.8 ± 2.2	22.7 ± 1.0	1.07 ± 0.07	50
In Vitro (unscraped)	23.4 ± 5.2	22.6 ± 1.4	1.23 ± 0.18	12

Values are mean ± S.E. Serosa is positive with respect to the mucosa.

The Jsc has been shown by Frantz and Rose (1968) to be equal to the net flux of Na^+ in the direction mucosa to serosa when normal Ringer bathes both surfaces of the membrane *in vitro*. In the present experiments the Jsc is considered to be a measure of the Na^+ transport rate. In some other experiments electrical measurements have been made simultaneously with the use of radioisotopes to give more complete information on ion fluxes. As explained in the review of the literature, the Jsc should be equal to the net flux of Na^+ if only Na^+ is transported across the membrane. To determine the net flux of Na^+ across an individual membrane, the serosal to mucosal flux was estimated by the technique described on page 21. The mucosal to serosal flux was measured by using ^{22}Na. The net flux was taken as the difference between the two unidirectional fluxes.

Net Na^+ Flux Equated to Jsc In Vitro

The following hypothetical example will serve to demonstrate for a membrane of high resistance (low passive flux) how the Jsc and net Na^+ flux can be equated over a one hour period with Ringer solutions bathing both surfaces of the crop membrane. The assumptions made in this calculation are:

1. the tissue reacts the same to ^{22}Na as to ^{23}Na;
2. the bathing medium is completely mixed by the stirring apparatus;

3. the injection of the isotopic Na^+ does not produce a significant Na^+ gradient across the membrane;
4. the specific activity of ^{22}Na is the same in the intracellular Na^+ transport pool as in the mucosal solution;
5. the passive Na^+ flux from serosa to mucosa is small and can be ignored (see Figure 5).

The amount of current (Jsc) used to null the PD to zero is calculated from the following data using the Faraday law of electrolysis according to Ussing (1951).

$$\frac{(Jsc)\ (t)}{(F)} = \text{quantity of electrons}$$

If:

Jsc = 80 μA of short-circuit current during a one hour period

t = time (3600 seconds)

F = Faraday (96500 μA sec/μEq)

$$\frac{(80\ (3600)}{(96500)} = 3\ \mu Eq \text{ of } e^-$$

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

$$\frac{(C)}{(A)}\ (D-B) = \mu Eq \text{ of } Na^+ \text{ transported from mucosal to serosal solution}$$

If:

A = 10000 cpm ^{22}Na in mucosal solution at t = 1 hr

B = 50 cpm ^{22}Na in serosal solution at t = 1 hr

C = 580 μEq Na^+ in mucosal and serosal solutions

D = 100 cpm ^{22}Na in serosal solution at t = 2 hr

$$\frac{(580)}{(10000)} \quad (100 - 50) = 3 \text{ μEq } Na^+$$

The number of electrons injected into the serosal chamber needed to null the PD is seen to be equal to the net Na^+ transport indicating that Na^+ is the actively transported ion.

Na^+ Depletion From the Mucosal Solution

The effect on Jsc and tissue R of a stepwise depletion of Na^+ from the mucosal solution of seven membranes *in vitro* is shown in Figure 7. The Jsc and PD measurements were made at Na^+ concentrations of 145 (Ringer solution) 14.5, 1.45 and 0.725 mEq/liter. The serosal surface was always bathed with Ringer solution. The PD and Jsc are reduced whether the Na^+ concentration is decreased by isosmotic choline substitution or by a hyposmotic water dilution. The average R immediately prior to the choline substitution sequence, while the mucosal surface was still bathed with normal Ringer solution, was 1.46 K ohms. After the Na^+ concentration had been reduced to 14.5 mEq/liter by choline substitution the R was 1.58 K ohms, which is not a significant change

Figure 7.--*In vitro* Jsc and PD values at four Na^+ concentrations (log scale) bathing the mucosal surface. Serosal surface was always bathed with Ringer solution. The Na^+ concentration was lowered first by choline substitution (dotted lines) and after return to normal Ringer it was lowered by water dilution (solid lines). The asterisk indicates that the $\bar{R}$ increased ($P < .01$) only with water dilution. The Jsc was decreased equally by choline substitution and water dilution. Exposed surface area was 2.54 cm^2. $\bar{R}$ values are given in K ohms. Brackets indicate S.E.

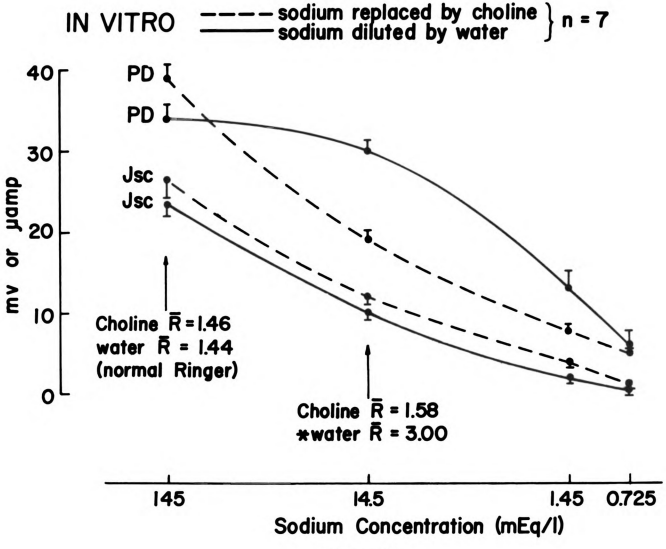
IN VITRO
sodium replaced by choline
sodium diluted by water
n = 7
40
30
20
10
0
mv or µamp
PD
PD
Jsc
Jsc
Choline R̄ = 1.46
water R̄ = 1.44
(normal Ringer)
Choline R̄ = 1.58
*water R̄ = 3.00
145
14.5
1.45
0.725
Sodium Concentration (mEq/l)

Figure 7

from the R in normal Ringer solution. Because of the small absolute values of the PD and Jsc, R values for Na^+ concentrations of 1.45 and 0.725 mEq/liter are not considered accurate enough to report. For instance, if the measured PD was in error by +1 mv due to imperfect pairing of electrodes the R would be calculated as much as 50 per cent too high at the Na^+ concentration of 0.725 mEq/liter.

After completion of the choline substitution sequence, Ringer solution again bathed the mucosal surface. After this return to control conditions the average R was 1.44 K ohms, a value quite close to the original control R of 1.46 K ohms. When the Na^+ concentration was reduced to 14.5 mEq/liter by water dilution the R increased significantly to 3.00 K ohms ($P < .01$).

The effect on PD and Jsc of a depletion of Na^+ from the mucosal solution was also studied in vivo. The PD and Jsc are seen to decrease (Figure 8 at low Na^+ concentrations as in the in vitro experiment. The average R of the membranes was not altered when Na^+ was depleted by choline substitution but it was again increased significantly ($P < .05$) by water dilution.

Effect of Hyperosmotic Ringer Solution

Ringer solution made hyperosmotic with sucrose, ethanol and dimethyl-sulfoxide (DMSO) bathing the mucosal

Figure 8.--*In vivo* Jsc and PD values at four Na^+ concentrations (log scale) bathing the mucosal surface. The Na^+ concentration was lowered first by choline substitution (dotted lines) and after return to normal Ringer it was lowered by water dilution (solid lines). The asterisk indicates that the R increased ($P < .05$) only with water dilution. The Jsc decreased more by choline substitution than by water dilution. Exposed surface area was 2.54 cm^2. R values are given in K ohms. Brackets indicate S.E.

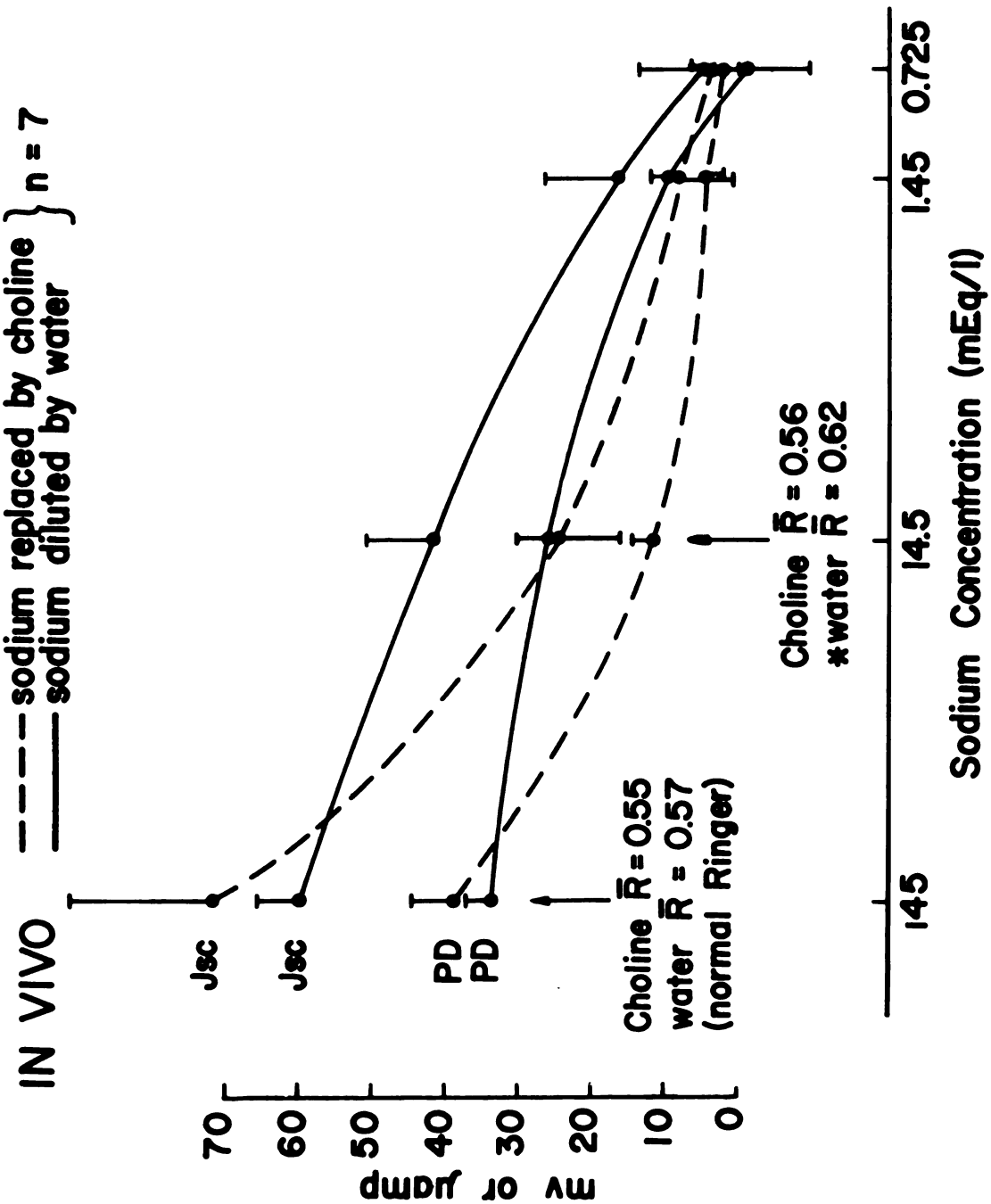
IN VIVO
sodium replaced by choline
sodium diluted by water
n = 7
Jsc
Jsc
PD
PD
Choline R̄ = 0.55
water R̄ = 0.57
(normal Ringer)
Choline R̄ = 0.56
*water R̄ = 0.62
mV or μAmp
70
60
50
40
30
20
10
0
145
14.5
1.45
0.725
Sodium Concentration (mEq/l)

Figure 8

surface of the crop membrane decreases the R and increases the Jsc *in vitro* but not *in vivo* (Table 2). In the present experiments *in vitro* the serosal surface was always bathed with Ringer solution. The extent of the decrease in R correlates with the Jsc increase ($r = -.32$, $P < .1$) when the hyperosmotic solutions were used *in vitro* (Figure 9).

Eight membranes *in vitro* were bathed initially with Ringer solution at both surfaces. Ringer solution made hyperosmotic with sucrose then bathed the membrane in the following sequence: (1) at only the mucosal surface; (2) at both the mucosal and serosal surfaces; (3) at only the serosal surface; (4) normal Ringer again bathed both surfaces. The results (Figure 10) indicate that a hyperosmotic solution at the mucosal surface results in a decrease in R (see also Table 2). When the hyperosmotic solution bathed both mucosal and serosal surfaces the R returned to about the control value. The hyperosmotic solution bathing only the serosal surface resulted in a further increase in R. When Ringer solution again bathed both surfaces of the crop, the R reached its highest value.

Resistance Values of the Crop in Vivo and In Vitro

The R of the crop membrane bathed with Ringer solution is 50-80 per cent less *in vivo* than *in vitro*

TABLE 2.--Effect on R and Jsc of crop membranes *in vivo* and *in vitro* by hyperosmotic mucosal bathing solutions.

Osmotic Agent Added	Condition	Control R K ohms	% Change R from Control	Control Jsc µA	% Change Jsc from Control	N	Time (min)
Sucrose	*in vivo*	0.38±0.13	-4.2±3.5	90.8±28.4	+15.6±9.9	7	57.8± 6.3
165 mOsm	*in vitro*	1.69±0.31	‡‡-10.4±2.8	21.5±4.00	*+25.4±8.3	8	47.1± 9.0
DMSO	*in vivo*	0.37±0.15	-2.4±4.1	110 ±14.7	-10 ±8.6	6	67.8±11.6
165 mOsm	*in vitro*	1.86±0.82	* -8.9±4.1	15.3±1.9	‡‡+23.0±8.5	10	50.3± 7.9
Ethanol	*in vitro*	1.40±0.50	* -8.8±4.6	16.1±1.6	‡‡+17.2±2.9	5	55.2±11.6
165 MOsM							
Ethanol	*in vivo*	0.51±0.14	+8.1±7.8	51.0±11.2	+3.94±8.0	6	42.8± 9.7
400 MOsM	*in vitro*	1.37±0.11	‡‡-15.4±3.3	13.3±1.7	‡‡+33.4 ±9.1	14	39.8±12.3

All values given as

mean ± S.E. *p < .05 ‡‡p < .01 N = number of observations

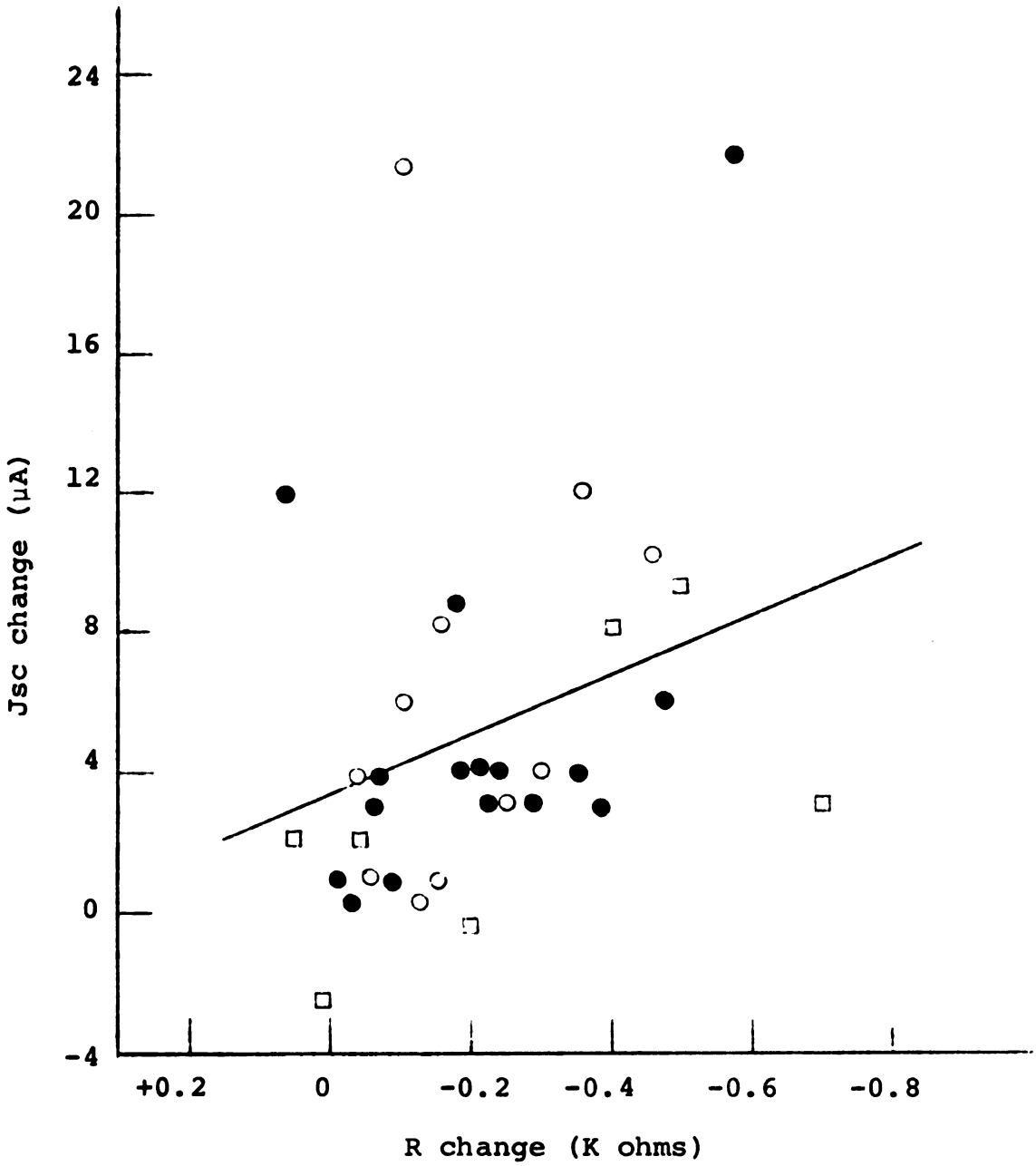

Figure 9.--Correlation of R decrease with Jsc increase when a hyperosmotic Ringer solution baths the mucosal surface of the crop in vitro ($r = -.32$, $p < .1$). □, DMSO: O, Sucrose; ●, ETOH. The line was fitted by the method of least squares.

(see Tables 1 and 4). This observation suggests that the membrane R changes when mounted *in vitro*. The R and Jsc values of 29 membranes were carefully observed during the initial 30 minute period of *in vitro* bathing. Figure 11 shows that most of these membranes increased in R during this period (average increase = 20 per cent; $P < .01$). There was a significant correlation ($r = -.47$; $P < .05$) between the increase in R and the decrease in Jsc. It is possible that the membrane undergoes structural (permeability) changes in response either to the process of removing it from the body of the bird or to the Ringer solution used. After 30 minutes *in vitro* the R of most membranes remained quite constant for several hours.

During the initial 30 minute period *in vivo* there were no consistent changes in the electrical characteristics of 20 membranes. There were frequently changes in the Jsc which were accompanied by similar increases or decreases of the PD. After 30 minutes *in vivo* a fairly steady state of PD and Jsc had usually developed.

Effect of a Cl^- Free Ringer Solution

A Cl^- free ($SO_4^=$) Ringer solution bathing the mucosal surface of the crop membrane *in vivo* and *in vitro* caused an increase in calculated R and an increase in Jsc (Table 3). The increase in R, a result of a greater PD, is expected if the membrane is less permeable

Figure 10.--Effect on crop R of a hyperosmotic solution. Sucrose-Ringer (465 mOsM) bathed the crop: at first arrow (extreme left), on mucosal surface; at second arrow, on mucosal and serosal surfaces; at third arrow, on serosal surface. At fourth arrow (extreme right) Ringer solution again bathed both surfaces. Asterisks beneath the arrows indicate the level of statistical significance of the change from the preceding value. Approximate time between arrows is 20 minutes. The initial R value was 1.69 K ohms. N = 8. Each bracket indicates the 95% confidence limit to be compared with the change in R (vertical line) on the immediate left of the bracket.

Figure 11.--Correlation between crop R change and Jsc change during initial 30 minute period *in vitro* ($r = -.47$, $P < .05$). Ringer solution bathed the membranes. The line was fitted by the method of least squares.

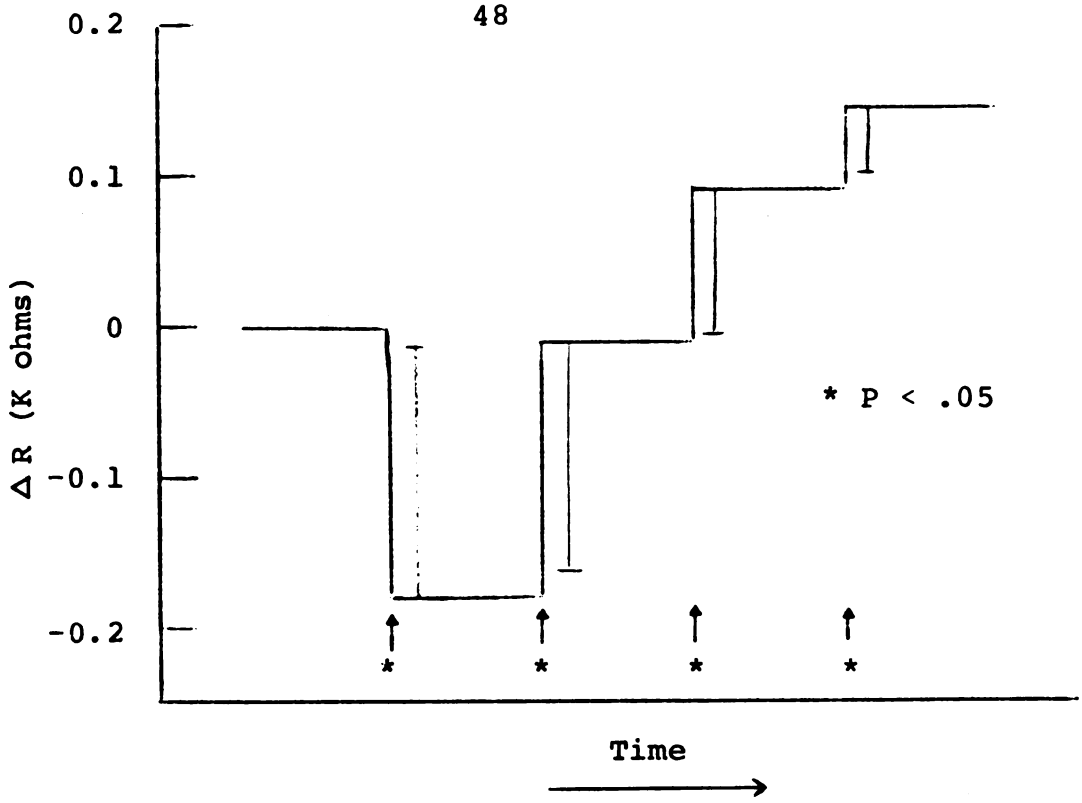

Figure 10

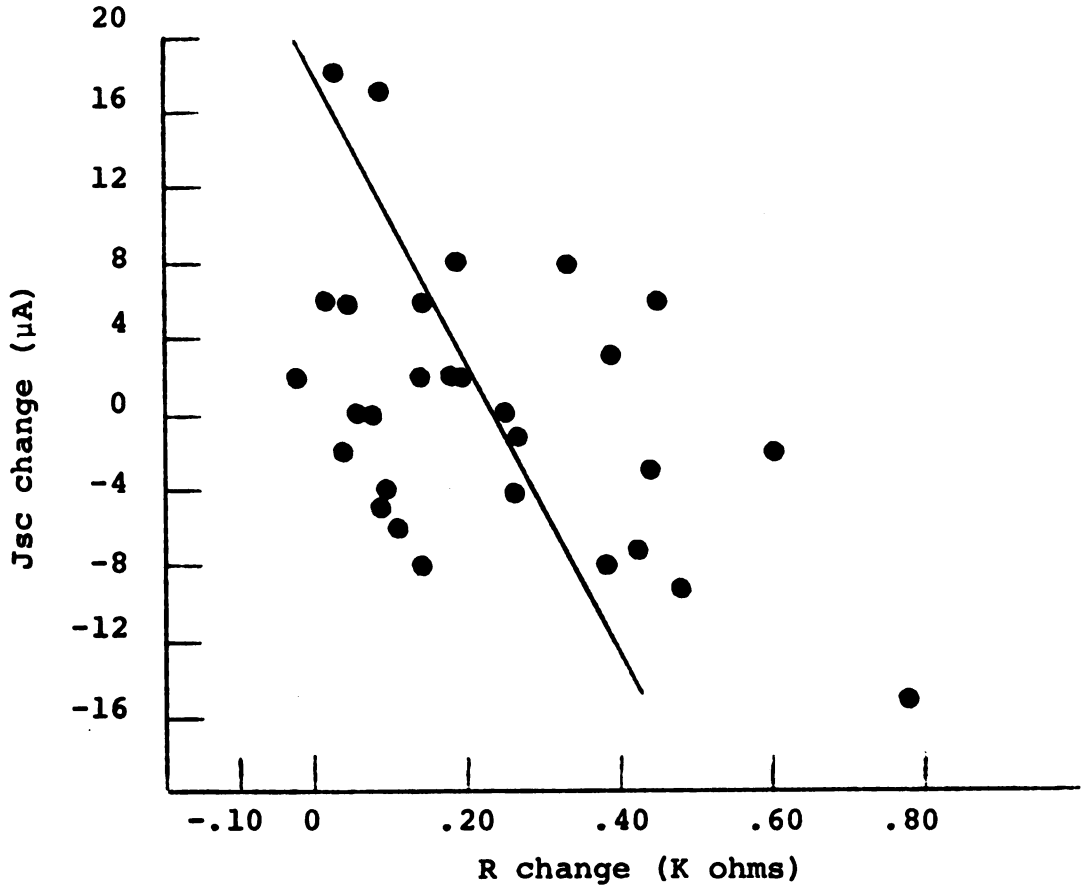

Figure 11

TABLE 3.--Effect on R and Jsc of crop membranes *in vivo* and *in vitro* by Cl^- free (SO_4) Ringer.

	% Change R	% Change Jsc
In Vivo		
Cl-Free Mucosal Soln.	‡+13.7±4.1(7)	‡+47.3±8.3(7)
In Vitro		
Cl-Free Mucosal Soln. Normal Ringer Serosal Soln.	‡+10.2±1.2(10)	‡41.9±11.4(10)
In Vitro		
Cl-Free Mucosal Soln. Cl-Free Serosal Soln.	‡+20.4±4.7(6)	-10.5±6.9(6)

Values are: mean + S.E. (N) ‡P < .01

to $SO_4^=$ than to Cl^-, since there will be less "shorting out" of the transmembrane potential in the presence of a non-diffusable anion. The increase in Jsc* was interpreted to be due to diffusion of Cl^- down the concentration gradient from serosal solution to mucosal solution thus contributing to the net flux of positive charge reaching the serosal solution. This possibility was tested by bathing both surfaces of six membranes *in vitro* with the Cl^- free Ringer solution thus eliminating the Cl^- concentration gradient. Under these conditions the membrane R again increased but the Jsc remained unchanged.

*The Jsc is a measure of the Na^+ transport only when equal solutions bathe each side of the membrane. With this ionic gradient set up across the membrane the Jsc will measure the summation of Na^+ transport and Cl^- diffusion.

Effect of $CuSO_4$

$CuSO_4$ ($6X10^{-4}M$) in the Ringer solution bathing the mucosal surface of the crop membrane *in vivo* and *in vitro* produced no change in membrane R but increased the Jsc (Figure 12). The increase in Jsc lasted an average of about one hour. The return to normal was only slightly faster if $CuSO_4$ was washed out of the chamber with several washes of Ringer solution (N = 3) at the peak of the response. The possibility that the change of Jsc was due to Cu^{++} altering the electrodes was tested *in vitro* by washing out the $CuSO_4$ from the mucosal solution and then replacing the potential sensing and short-circuiting electrodes of this solution with electrodes which had not been in contact with $CuSO_4$ (N = 3). Since no difference in the end of the stimulatory phase (starting at t = 9 minutes) was measured with the replacement electrodes which had never been exposed to Cu^{++}, it is assumed that the Cu^{++} effect was on the membrane and not on the electrodes.

$CuSO_4$ ($6X10^{-4}M$) in the Ringer solution bathing the serosal surface resulted in a gradual reduction of the Jsc to zero. An equal concentration of Na_2SO_4 in the Ringer solution bathing either the mucosal or serosal surface had no measurable effect on the Jsc or R.

Figure 12.--Effect on crop membrane R and Jsc *in vivo* and *in vitro* by $CuSO_4$ ($6X10^{-4}M$ final concentration) in Ringer solution bathing the mucosal surface at time zero. The X's represent the Jsc *in vitro* when the mucosal solution is replaced by Ringer solution at peak of $CuSO_4$ effect. Open circles represent Jsc values when fresh electrodes are used in the mucosal Ringer solution from which $CuSO_4$ has been washed out. Jsc was increased *in vivo* ($P < .01$) and *in vitro* ($P < .05$), but there was no significant change of R *in vivo* or *in vitro*. Exposed surface area was 2.54 cm^2.

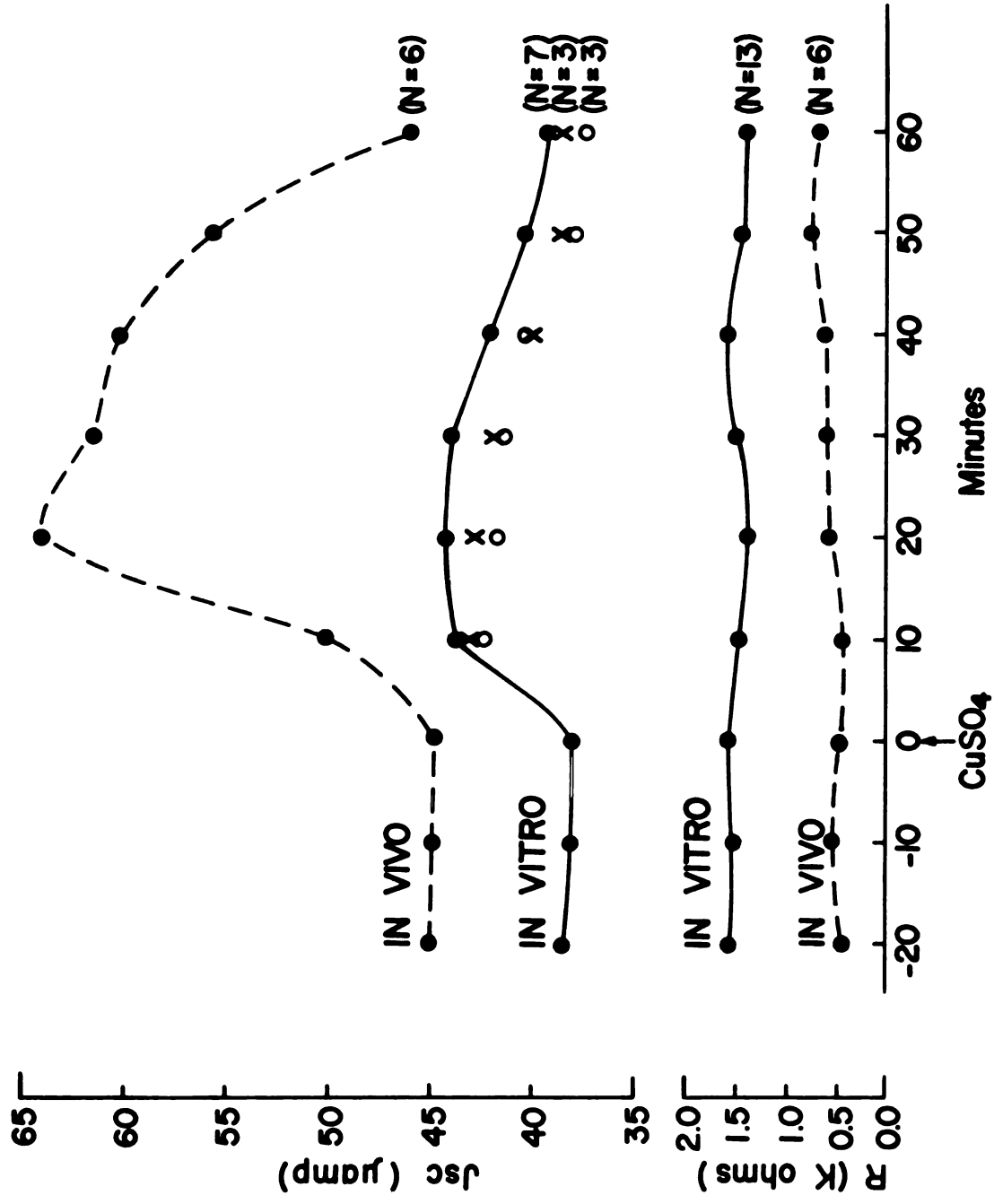
65
60
55
50
45
40
35
Jsc (μamp)
IN VIVO
IN VITRO
(N=6)
(N=7)
(N=3)
(N=3)
2.0
1.5
1.0
0.5
0.0
R (K ohms)
IN VITRO
IN VIVO
(N=13)
(N=6)
-20
-10
0
10
20
30
40
50
60
CuSO4
Minutes

Figure 12

Ion Regulation by the Crop

The capability of the crop membrane to act as a component of the pigeon's ion regulation system has been evaluated in the following manner. Birds were assumed to be in a steady state of Na^+ balance (i.e., Na^+ intake/day = Na^+ loss/day) and their total Na+ intake was estimated by measuring the quantity of food eaten over a four day period. The Na^+ content of the feed was estimated from values reported by Ewing (1963). This method of estimating whole body Na^+ intake assumes that all Na^+ ingested is eventually absorbed. After the fourth day of estimating Na^+ consumption the bird was prepared to measure the Jsc *in vivo*.

The rate of Na^+ transport by the membrane was estimated by measuring the Jsc/2.54 cm^2. The transport rate of the area measured is assumed to be representative of the whole crop surface. Since the volume of the crop was known by the amount of Ringer solution it held, the surface area could be estimated by considering it to be of spherical shape. The total Jsc was calculated from the value per 2.54 cm^2 and the estimated surface area and converted to mEq Na^+/day. In the two birds tested the upper limit of Na^+ absorption by the crop appears to be 55-75 per cent as great as the ingested Na^+ (Table 4). It thus seems that the crop membrane *in vivo* has the ability to transport sufficient quantities of salt to

TABLE 4.--Estimated ability of the crop membrane to absorb Na^+.

	Jsc/2.54 cm^2	Na^+ absorbed by crop/day†	Feed Consumption per day	Total Na^+ Absorption per day††
Bird 1	80 μA	1.5 mEq	48 g	2.6 mEq
Bird 2	120 μA	2.2 mEq	54 g	2.9 mEq

†Calculated from Jsc value and estimated crop surface area.

††Estimated from feed consumption.

assign to it tentatively a role in mineral absorption. The actual uptake of salt by the crop *in situ* will depend on how much salt is available to the tissue during the several hour period that the feed remains stored in the crop lumen. The appropriate values of luminal Na^+ concentration are not currently available.

Effect of Ca^{++} on the Crop

Six crop membranes were initially bathed *in vitro* with a Ringer solution containing Ca^{++} (4.3 mEq/l). After a steady state condition of Jsc and Pd had been established a Ca^{++} free (0.3 mEq/l) Ringer solution was substituted at both the mucosal and serosal surfaces (Figure 13). This solution resulted in a reversible 37 per cent increase in Jsc and a seven per cent decrease in R. This combination of responses indicates that the presence of Ca^{++} results

in a decreased rate of passive ion movement through the tissue.

Two crop membranes in vivo were initially bathed on the luminal surface with a Ca^{++} free Ringer solution. During steady state conditions of Jsc and R, Ca^{++} was added to the crop lumen to obtain a final concentration of 4.3 mEq/liter (Figure 14). There was a resultant decrease in Jsc and an increase in resistance of each membrane. The effects were reversed when fresh Ca^{++} free Ringer again bathed the luminal surface.

Cation Replacement Studies

The replacement of Na^+ by Li^+ as the primary cation in the mucosal solution in vivo resulted in less of a depression of Jsc than when Na^+ was depleted by choline substitution (Figure 15). These data suggest that in the absence of Na^+, Li^+ may be actively transported from the mucosal to the serosal surface. However, under these conditions there is a concentration gradient of Na^+ from the serosal surface to the mucosal solution and an approximately equal gradient of Li^+ in the opposite direction. If the crop membrane is more permeable to Li^+ than to Na^+, the net flux of Li^+ will exceed that of Na^+ and the serosal surface will become positively charged. The short-circuiting recorder, when activated, will null the entire transmembrane PD to zero, and the Jsc indicated

Figure 13.--Effect of Ca^{++} free Ringer solution bathing both surfaces of the crop *in vitro*. Initial R and Jsc values are with a Ca^{++} concentration of 4.3 mEq/liter in the Ringer solution. When a Ca^{++} free Ringer solution bathed both surfaces of the membrane (first arrow) there was a 37 per cent increase in Jsc and a 7 per cent decrease in R. At the second arrow the Ca^{++} Ringer solution again bathed the membrane. Approximate time to reach new steady state conditions after depletion or addition of Ca^{++} was 20 minutes. Changes of R and Jsc were significant at $P < .05$ level. $N = 6$.

Figure 14.--Effect of Ca^{++} added to the mucosal bathing solution *in vivo*. Initial R and Jsc values are with a Ca^{++} free Ringer solution. When Ca^{++} was added (first arrow) to a final concentration of 4.3 mEq/l there was an average increase in R of 19 per cent and an average decrease in Jsc of 26 per cent. At the second arrow the Ca^{++} free Ringer again bathed the lumen. Approximate time to reach a new steady state condition after addition or depletion of Ca^{++} was 30 minutes.

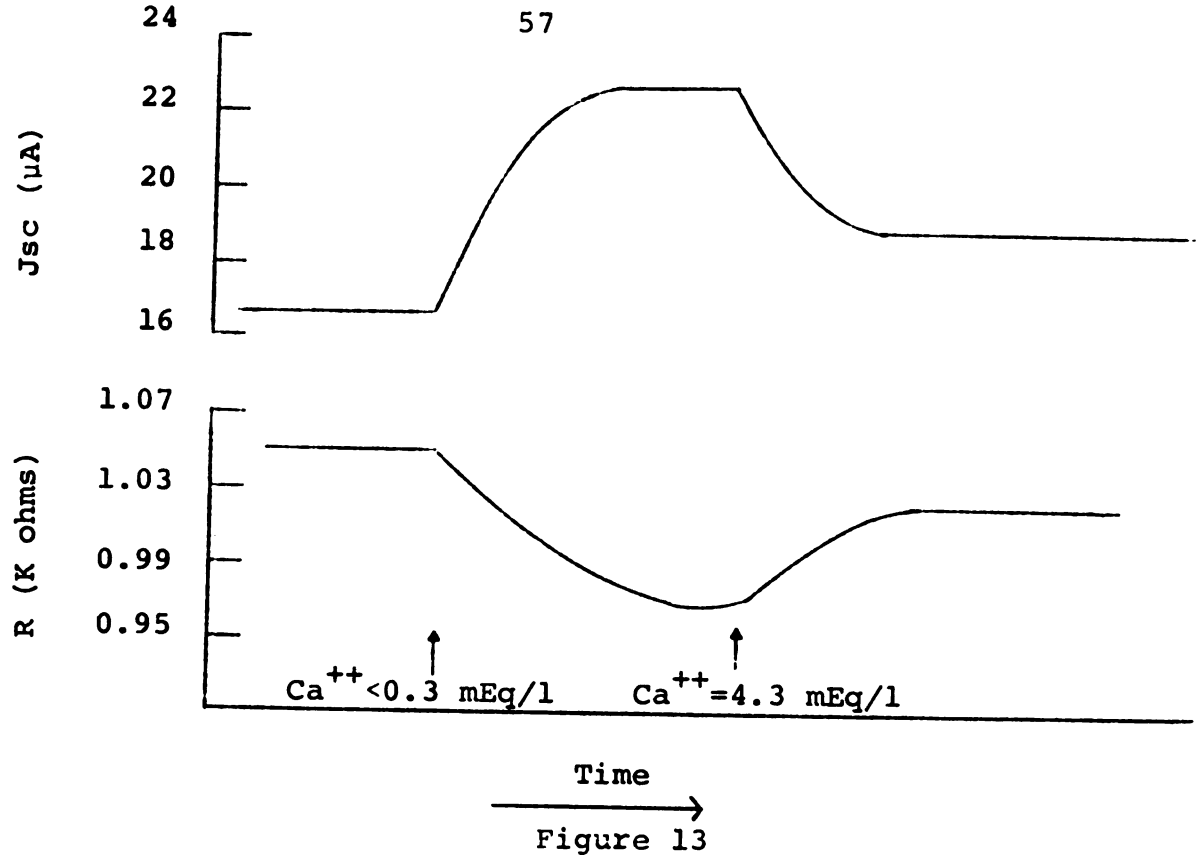

Figure 13

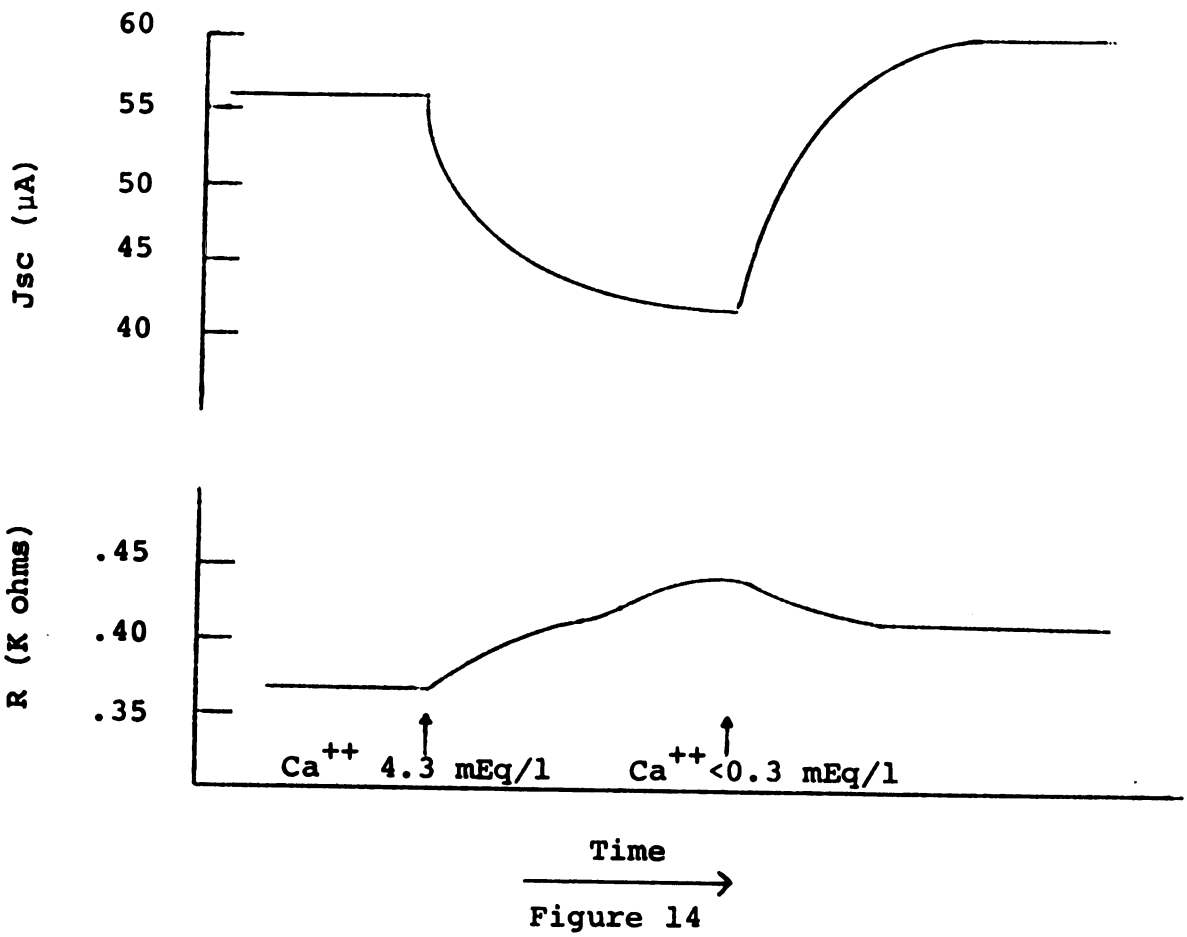

Figure 14

will be a measure of the net ionic flux due both to the active transport process and diffusion.

The possibility that a diffusion potential contributes to the Jsc under these conditions was tested *in vitro*. Seven membranes were bathed in Ringer solution until a steady state of Jsc and PD was recorded. When Na^+ was replaced by Li^+ and in the mucosal solution the Jsc decreased by 17 per cent, a result similar to that in the *in vivo* experiment. Fresh Li^+ Ringer was then used to bathe both surfaces of the membrane, thus eliminating transmembrane ionic concentration gradients. There was a further 66 per cent decrease in the Jsc. These results indicate that Li^+ cannot substitute for Na^+ in the active transport mechanism.

K^+ Replacement by Rb^+ and Cs^+

The effect on the Jsc of depleting K^+ from the serosal solution is shown in Figure 16. The decrease in Jsc is unaffected by either Rb^+ or Cs^+ replacement. In one experiment using Rb^+ in place of K^+ in the serosal solution the membrane PD was continuously recorded. Shortly after Rb^+ was added to the serosal solution a rhythmical variation in the transmembrane PD developed (Figure 17). The initial amplitude of variation was about 1 mv, and it damped to about 0.2 mv after nine oscillations. The average frequency was 0.11 oscillations

Figure 15.--Effect on Jsc when Li^+ substitutes for Na^+ in the Ringer solution. The Li^+ substitution is made at the first arrow. After a transitory increase, the Jsc levels off only slightly below the *in vivo* control value. The Jsc *in vitro* shows the same response when Li^+ substitutes for Na^+ in only the mucosal solution. When Li^+ substitutes for Na^+ in both solutions *in vitro* (second arrow) the Jsc is reduced to 25 per cent of its control value. Dotted lines show the effect of replacing Na^+ of the mucosal solution by choline.

Figure 16.--Effect on Jsc when Cs^+ or Rb^+ replaces K^+ in the serosal solution. The transitory increase in the Jsc may be due to the greater diffusion gradient of K^+ from the membrane to the serosal solution. The presence of either replacement ion does not prevent the decrease in Jsc. Solid line represents K^+ depletion with no replacement (N = 3). Circles represent Cs^+ replacement (N = 3). □'s represent Rb^+ replacement (n = 3).

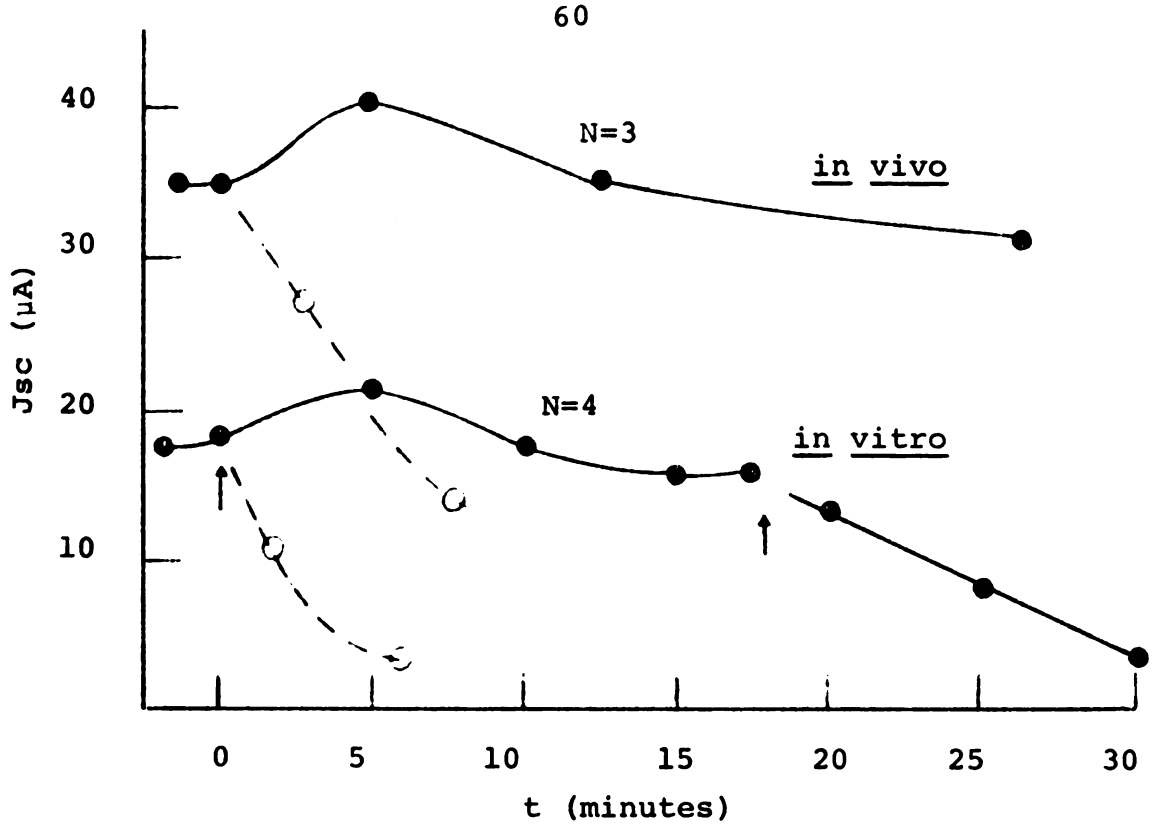

Figure 15

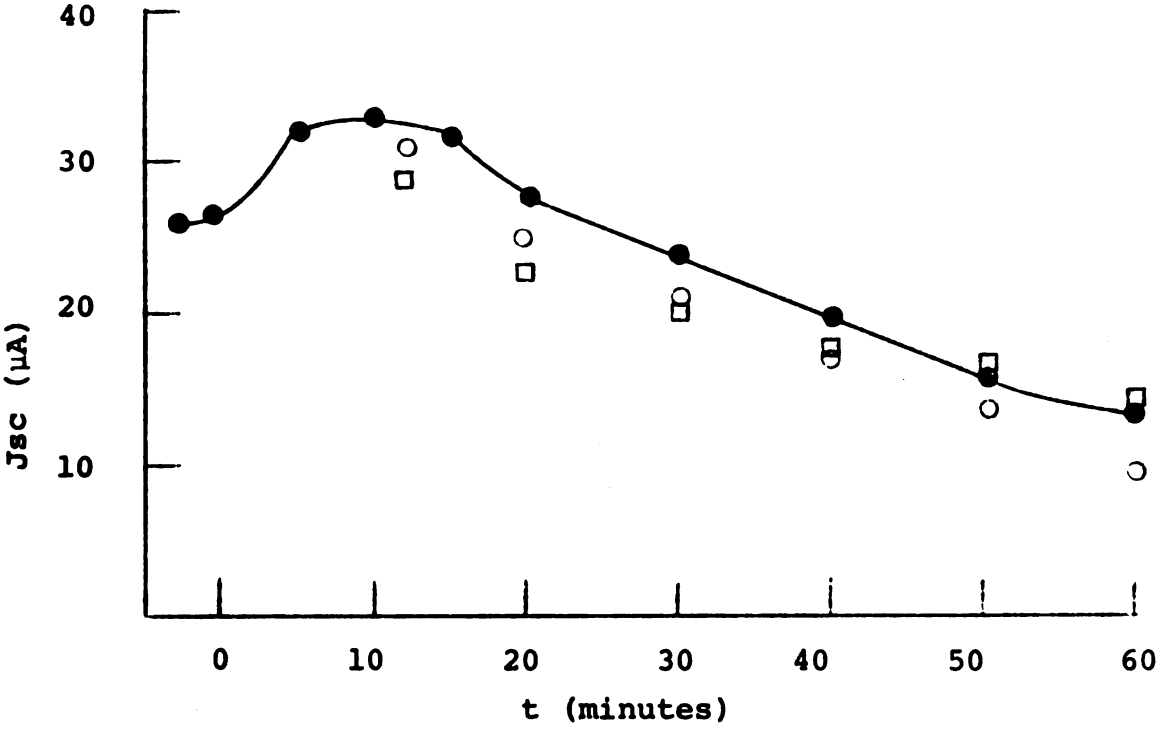

Figure 16

per minute. When Ringer solution again bathed the serosal surface the oscillation damped out after three cycles.

pH Studies

The crop Jsc *in vitro* was found to be quite independent of the mucosal solution pH. Ringer solutions adjusted to pH 6.0 using HCl or 9.0 using NaOH bathing the mucosal surface for 20 minutes had no effect. The pH of the serosal solution was adjusted at 0.3 pH unit intervals through the range 6.8-8.3. It was found that the pH must be within the range 7.4-7.7 for maximal Na^+ transport activity. A pH outside this range usually began to reduce the Jsc within five minutes.

Hormone and Drug Studies

Injection of the parasympathomimetic agent carbachol chloride (lentin, 3 ug/Kg) intramuscularly to preparations *in vivo* resulted in an average increase in Jsc of 67.4 $\mu A/2.54\ cm^2$ ($P < .01$) (Figure 18). The PD increased proportionately so there was no significant change in R. The peak Jsc came at 27 minutes and the effect was essentially over after 60 minutes.

Rehm (1968) has suggested on the basis of theoretical work that blood flow through a tissue *in vivo* may prevent the potential across the active tissue from being completely nulled to zero. He suggested that the measured Jsc would

Figure 17.--Rhythmical variations of the *in vitro* crop PD induced by Rb^+ replacement for K^+ in the serosal solution. At the first arrow rubidium-Ringer was used at the serosal surface; at the second arrow normal Ringer solution was again used at the serosal surface. Single observation.

Figure 18.--Effect of carbachol chloride (lentin) on the crop Jsc. *In vivo* there was a 67 per cent increase ($P < .01$). There was no measurable effect *in vitro*. Base level Jsc was 59 μA *in vivo* and 28 μA *in vitro* (N = 6 *in vivo* and *in vitro*).

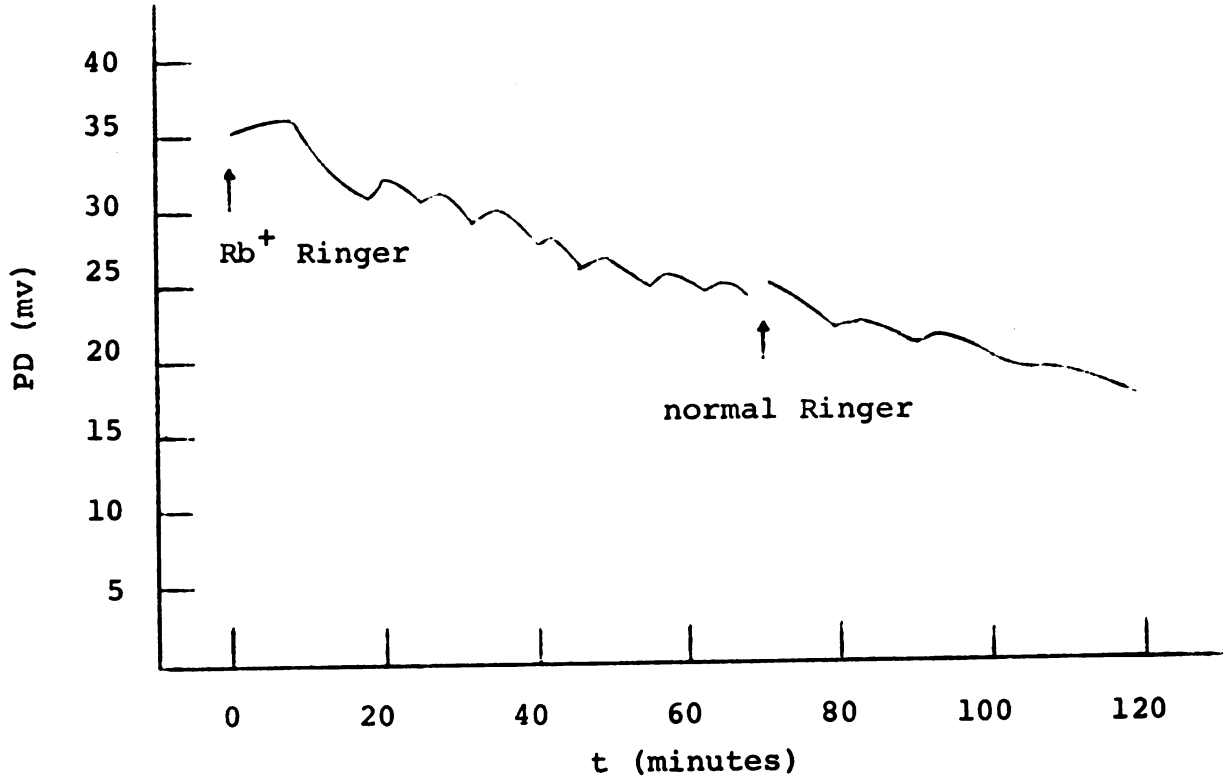

Figure 17

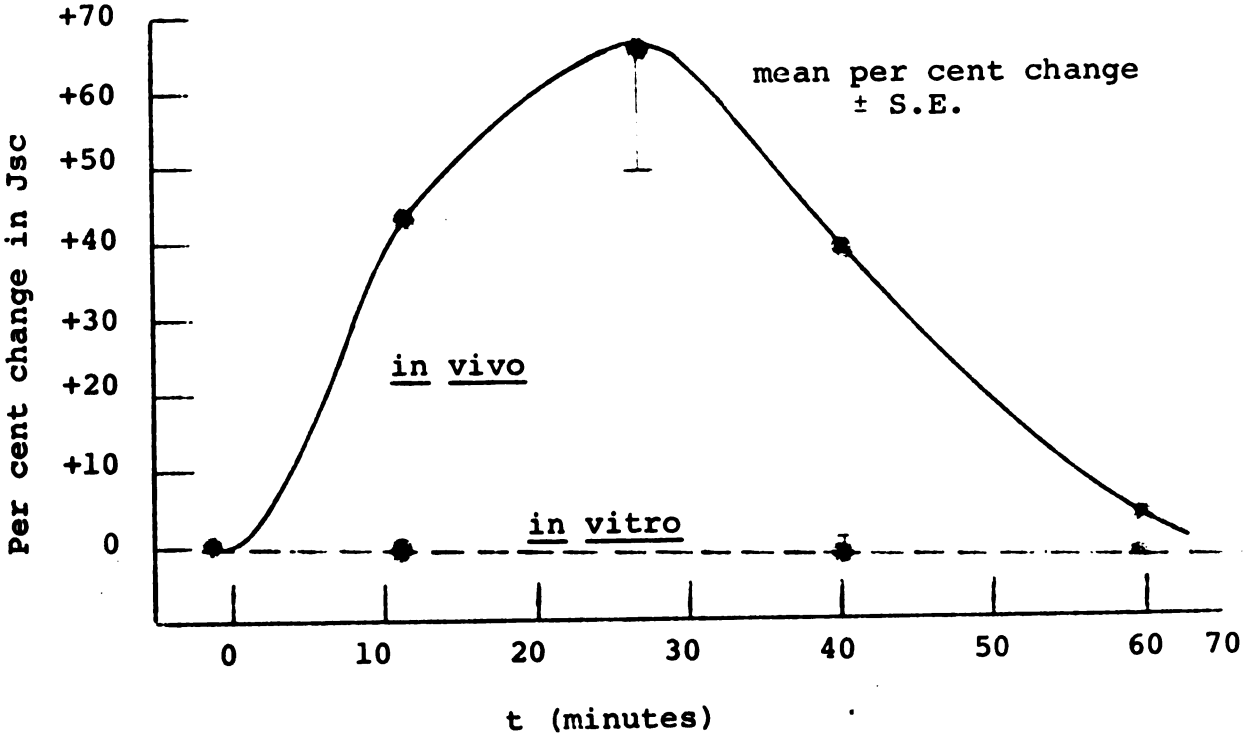

Figure 18

therefore be less than the true Jsc. It may be that the primary cholinergic effect of carbachol chloride on the crop membrane *in vivo* is a reduction of blood flow which results in slower removal of accumulated ions and therefore a greater Jsc. The lack of an effect on the Jsc or PD when carbachol chloride bathed the serosal surface of the crop *in vitro* (0.75 mg/liter) supports the theory that the drug has no direct effect on the transport mechanism (Figure 18).

Epinephrine (5 µg/Kg) injected im to preparations *in vivo* resulted in a small increase in the Jsc of two membranes and in no response in two others. Epinephrine injected into the serosal bathing solution (1.5 mg/liter) of three membranes *in vitro* resulted in no measurable change in the PD or Jsc in a 30 minute period. The response *in vivo* may have been due to an effect on the cardiovascular system as in the case of the cholinergic agent.

The effect of prolactin on the electrical characteristics of the crop was evaluated in three different ways: (1) Prolactin (25 µg) was injected intradermally over one lobe of 10 birds. The other lobe received an equal amount of fetal calf serum. After 24 hours the birds were killed and one sample of tissue from each lobe of the crop was mounted *in vitro*. (2) Prolactin (0.4 mg/Kg) was injected into the pectoral muscle of five birds and

five other birds injected with calf serum served as a control. After 24 hours the birds were killed and the crop membranes were mounted *in vitro*. (3) Prolactin was applied directly to the serosal surface of three crop membranes *in vivo* as the PD and Jsc were alternately measured. The application of prolactin by the three techniques used did not appear to affect the membrane PD or Jsc (Table 5). The Jsc was still equal to net Na^+ flux under conditions *in vitro*.

TABLE 5.--Effect of prolactin on crop Jsc and PD.

	Prolactin (25 µg) intradermal	Prolactin (0.4 mg/Kg) im.	Prolactin (0.2 mg) *in vivo*
% change Jsc	+20.2 ± 15.7	+3.4 ± 9.2	-6.8 ± 4.7
% change PD	+12.8 ± 17.0	-4.6 ± 7.2	-8.2 ± 7.6
	(10)	(5)	(3)

Values are mean ±S.E. The number of treated birds is in parentheses.

Pitressin (2 IU/ml) was added to the serosal solution of the crop *in vitro* and injected im (2 IU) to the pigeon during experiments *in vivo*. There was no measurable effect on the membrane PD or Jsc in either case (Table 6).

The effect of aldosterone on the Jsc of the crop membrane was evaluated in two ways. It was injected im

TABLE 6.--Effect of pitressin on crop Jsc and PD.

	Pitressin (? IU) *in vivo* (im)	Pitressin (2 IU/ml) *in vitro*
% change Jsc after one hour	+3.6 ± 2.1	-5.8 ± 5.2
	N = 4	N = 5

Values are mean ± S.E.

to seven birds and the Jsc was measured 15 hours later *in vitro*. An equal volume of Ringer solution was injected into seven other birds that served as controls. Aldosterone was also injected into the serosal bathing solution ($1X10^{-6}M$) during five *in vitro* experiments. There was no measurable effect of aldosterone by either application (Table 7).

TABLE 7.--Effect of aldosterone on crop Jsc.

	Aldosterone (40 µg/Kg) im	Aldosterone (360 µg/liter) *in vitro*
% change in Jsc	+10.4 ± 14.7	-8.7 ± 5.6
	N = 7	N = 5

Values are mean ± S.E.

Lack of Interaction Between Na^+ Transport and Amino Acid or Sugar Transport

The presence of either amino acids or sugars (10 mM final concentration) in the mucosal bathing solution in vitro resulted in no increase in Jsc over a 30 minute period. These results indicate that the active Na^+ transport mechanism does not interact with the movement of amino acids or sugars (Table 8).

TABLE 8.--Effect of amino acids and sugars on the crop Jsc *in vitro*.

	Fructose (10 mM)	Glucose (10 mM)	Alanine (10 mM)	Leucine (10 mM)
% change in Jsc	+2.0±3.8	-8.7±11.2	-4.8±10.7	-2.7±7.4

Values are mean ± S.E. N = 4 for each experiment.

Decrease of the Jsc In Vivo when Death of the Pigeon Occurs

When a steady value of Jsc of an *in vivo* preparation was measured the bird was killed by cervical dislocation in order to follow the time course of the decay of the Jsc (Table 9).

TABLE 9.--Decrease of Jsc *in vivo* following sudden death of the pigeon.

	Time After Death (Minutes)							
	0	10	20	40	60	80	100	120
Jsc (μA)	106	95	80	51	29	17	9	4

Seasonal Variations of the Crop Jsc

During 1968 the average Jsc of crop membranes *in vitro* was highest during the summer months. The data are presented in Table 10.

TABLE 10.--Seasonal variations of the crop Jsc *in vitro*.

	January-April	May-August	September-December
Jsc (μA)	10.4 ± 2.3	29.6 ± 5.4	12.6 ± 2.5
	N = 27	N = 33	N = 37

Values are mean ± S.E.

CHAPTER V

DISCUSSION

Evidence for Active Transport of Na^+ in Vivo

A statistical demonstration of an active transport process under *in vivo* conditions is difficult to make. The present study has not included the use of radioactive isotopes to estimate the amount of Na^+ transported *in vivo* by the crop membrane. Rather, the electrical characteristics of the crop have been examined both *in vivo* and *in vitro*, and the results of these two series of experiments are compared.

The crop membrane *in vitro* has previously been demonstrated by Frantz and Rose (1968) to satisfy the criteria listed by Brown (1965) of an active ion transport mechanism. The specific criteria are listed below along with the current evidence for an active Na^+ transport process *in vivo* in the pigeon crop mucosa:

(1) "The force is located within the membrane."

The force which develops a transmembrane PD across the crop is evidently located in the membrane itself.

The PD is not a consequence of a passive diffusion gradient since the Ringer solution has nearly the same osmotic and ionic composition as avian blood (Prosser and Brown, 1961).

(2) "The force directly influences particle motion"

The present study has not demonstrated that this criterion is met by the crop membrane *in vivo*. One method of demonstrating that this condition is met under *in vivo* conditions is to compare the membrane Jsc with the net flux of Na^+ as was done in the *in vitro* study. This could be done by using the method Curran and Solomon (1957) have used to estimate the net ion fluxes through the ileum under *in vivo* conditions.

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

There is no significant chemical gradient of Na^+ across the crop membrane when Ringer solution bathes the mucosal surface. Since the serosal surface is positive with respect to the mucosal solution (Table 1), a movement of Na^+ from the mucosal solution must be against an electrical gradient. Therefore the free energy of Na^+ would be increased as it crossed the membrane.

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

This criterion is unspecifically demonstrated by the crop when death of the animal occurs during an *in vivo*

preparation (Table 9). The slow decline of the Jsc is indicative of a loss of metabolic energy which normally would have been supplied by substrates delivered through the blood. No studies have been done *in vivo* with metabolic inhibitors; the problem of delivering an inhibitor to only the crop transport mechanism has not been solved.

Perhaps the strongest evidence that a Na^+ transport process exists in the crop *in vivo* is the decrease of the Jsc, similar to the *in vitro* experiment, when the Na^+ concentration of the mucosal solution is decreased (Figure 8). If only Na^+ transport is the cause of the Jsc, then a lack of Na^+ available to the transport mechanism from the mucosal solution would result in a reduction of the Jsc.

Comparison of Tissue Resistance In Vivo and In Vitro

Brown (1962) reported that the resistance of the frog skin *in vivo* was lower than *in vitro*. He speculated that there was a decrease in the permeability characteristics of the skin in response to the *in vitro* environmental conditions. The crop membrane permeability may also have changed when the membrane was taken out of the bird as evidenced by the lower resistance values *in vivo* than in either the scraped or unscraped crop *in vitro* (Table 1).

The degree of increased R of the individual crop membranes correlated inversely with the changes in the Jsc of the membranes during the initial 30 minute period *in vitro* (Figure 11). The decreased ionic permeability of the membrane may result in reduced values of the Jsc either due to slower arrival of Na^+ from the mucosal solution to the pump site or due to slower diffusion from the pump site to the serosal solution. The comparison of *in vivo* and *in vitro* observations on both frog skin and crop tissue illustrates the inadequacy of examining a biological tissue only under *in vitro* conditions.

Effect of Hyposmotic Solutions on Tissue Resistance

Water-diluted (hyposmotic) Ringer solutions bathing the mucosal surface of the crop membrane caused an increase in R of 9 per cent (0.05 K ohms) *in vivo* and 109 per cent (1.56 K ohms) *in vitro*. Brown (1962) reported that water-diluted Ringer solutions bathing the outside surface of the frog skin *in vivo* and *in vitro* resulted in an increase in resistance (decrease in conductivity). He attributed the change in R to the low concentration of Cl^- which would be available to diffuse through the skin and short out the membrane PD.

However, a low Cl^- concentration is not the only possible explanation of an increase in resistance under these conditions. The work of Leb, Hoshiko and Lindley

(1965) suggests that a hyposmotic Ringer solution bathing the mucosal surface of amphibian bladders results in higher transmembrane potentials, and therefore, if the Jsc remained constant, a higher R. Biber, Chez and Curran (1966) using hyposmotic solutions to bathe the outside surface of frog skins reported a marked reduction in Cl^- permeability from the inside to the outside surface. A hyposmotic Ringer solution may swell the tissue cells by osmosis thus limiting the extracellular shunt pathways available for passive ion diffusion. The elimination of passive Cl^- diffusion down the electrical gradient would tend to raise the PD, thus increasing the R.

Rose and Frantz (1967) have reported that the R of individual crop membranes bathed *in vitro* in Ringer solution does correlate inversely with passive fluxes of ^{36}Cl and ^{22}Na. The hyposmotic, low Cl^- solution bathing the mucosal surface of the crop membrane increases the R due either to the low Cl^- concentration or to the hyposmotic property.

The relative contribution of these two factors can be evaluated by a comparison of the R effects of hyposmotic, low Cl^- solutions (Figures 7 and 8) with the effects of the isosmotic, low Cl^- solution (Table 3). Under *in vivo* conditions the R change (Figure 8, solid lines, 9 per cent increase) in response to a hyposmotic, low Cl^- solution

was no greater than the R change (Table 3, 13.7 per cent increase) with an isosmotic, low Cl^- solution. *In vitro*, however, the R change (Figure 7, solid lines, 109 per cent increase) was much larger in response to the hyposmotic, low Cl^- solution than to the isosmotic, low Cl^- solution (Table 3, 10.2 per cent increase). These results indicate that a hyposmotic solution has little effect on the membrane permeability characteristics *in vivo* but a pronounced effect *in vitro*. The lack of a response *in vivo* may be due to the ability of the animal's intact circulatory system to buffer changes in tissue osmolarity, thus preventing much effect on the permeability characteristics.

The less pronounced decrease of Jsc and PD *in vivo* when the Na^+ concentration is reduced by water dilution rather than by choline substitution may be due to diffusion of Cl^- from the membrane into the water-diluted (low Cl^-) Ringer solution in the crop lumen. This effect is not observed *in vitro*, perhaps because the restriction of intercellular pathways (high membrane R) limits Cl^- diffusion.

Effect of Hyperosmotic Solutions on Tissue Resistance

Ussing and Windhager (1964) and Lindley, Hoshiko and Leb (1964) have shown that hyperosmotic solutions bathing the outside of the frog skin increase the passive leakage of ions presumably due to dehydration and shrinkage of the

tissue cells and consequent opening of the seal between the cells of the outermost layer. A decrease in R also resulted when the crop membrane *in vitro* was bathed with solutions made hyperosmotic by using sucrose, ethanol and DMSO (Table 2). Our inability to demonstrate statistical significance of this effect *in vivo* may be due to buffering of the tissue osmolarity by the circulating blood, as in the hyposmotic experiment (Figures 7 and 8).

The hyperosmotic solutions also resulted in an increase in the crop Jsc *in vitro*. The correlation between the R decrease and the Jsc increase (Figure 9) indicates that these are not independent responses. The Jsc may increase because Na^+ can pass more easily from the mucosal solution through the extracellular spaces of the s. disjunctum to the Na transport cells. The R and Jsc changes would correlate inversely because the degree of each would depend on the extent of opening of the extracellular spaces. Alternatively, the Jsc may increase due to shrinkage of the transport cells themselves. Since intracellular Na^+ would not leave the cell as rapidly as water, its concentration would increase. The transport mechanism could become more saturated and an increase in the transport rate would occur. If a significant barrier to ion diffusion were located in the cells of the transport layer, then shrinkage of those cells would correlate with R changes.

DMSO has been reported by Klingman (1965a, 1965b) to rapidly penetrate the skin while at the same time facilitating entry of other substances. It has thus been suggested that DMSO alters the permeability of certain membranes by a specific chemical effect. DMSO bathing the mucosal surface of the crop in vitro resulted in changes in electrical characteristics similar to those observed from the same concentration of sucrose and thus can be explained on the basis of osmotically induced changes of the membrane R. The previous report by Morain, Replogle and Curran (1966) that DMSO changes the permeability of isolated frog skin also indicated that the effect was primarily an osmotic one. Israel and Kalant (1963) reported that ethanol bathing the outside surface of frog skin inhibits the Jsc. They suggested that a specific chemical mechanism of action may exist. In the present experiments the effects of ethanol bathing the mucosal surface of the pigeon crop can be explained in terms of its osmotic activity (Table 2).

The decreased R when a hyperosmotic solution bathed the mucosal surface of the crop in vitro (Table 2), was attributed to dehydration and shrinkage of the tissue cells which would result in a larger volume of the extracellular channels through which ions pass. Since Cl^- could more easily follow the actively transported Na^+ across the membrane there would not be as great a

separation of charge and the PD would be reduced. The R responses when a hyperosmotic solution bathed both surfaces or only the serosal surface are not as easily accounted for. The results in Figure 10, however, are qualitatively similar to those of Lindley, Hoshiko and Leb's experiment (1964) using hyperosmotic solutions on frog skin. These authors suggest that changes in "shunting" of ions crossing the membrane is one way hyperosmotic solutions may decrease membrane potentials.

Changes in "shunting" of Cl^- could account for the increased membrane permeability in Figure 10 (first arrow) as described above. However, it is difficult to explain the subsequent R increases in this figure as being due to changes in extracellular shunt paths in response to the hyperosmotic solution. The information needed to decide what causes the R increases would be the result of a much more intensive study than the present one.

Effect of Ca^{++} on Jsc and Resistance

The presence of Ca^{++} in the Ringer solution *in vitro* and *in vivo* is associated with a higher R and a lower Jsc of the crop membrane. Curran *et al.* (1963) have reported that Ca^{++} (11.3 mM) decreases the Na^+ permeability of the outer membrane of the frog skin. This results in a reduction of the Na^+ pool of the transporting system which reduces the Na^+ transport rate. Curran and Gill

(1962) reported that a lack of Ca^{++} in the outside bathing solution causes the ionic permeability of the skin to increase. These reports are consistent with the theory that Ca^{++} helps to regulate membrane permeability by affecting pore sites. A decreased permeability of the crop membrane to Cl^- would result in a greater separation of charge (Na^+ from Cl^-) and a higher R would be calculated. A decreased permeability of the membrane to Na^+ may restrict the entry of Na^+ to the transport mechanism thus causing it to be less saturated.

Effect of Cu^{++} on Jsc and Resistance

The effect of $CuSO_4$ on the crop is different from its reported effect on other biological tissues. Cu^{++} did not affect the frog skin Jsc but did increase the resistance and PD according to Ussing (1949). This effect on the frog skin resistance was explained by Ussing as a decreased permeability of the membrane to Cl^-. In the present experiment (Figure 12) there were no consistent changes in membrane R but an increase in the Jsc of 43 per cent *in vivo* and 19 per cent *in vitro*. The maximum effect on the frog skin came after one to two hours while the Cu^{++} effect on the crop membrane reached a peak in 20 minutes and was essentially over after one hour. The specific response may be an easier passage of Na^+ from the mucosal solution into the crop transport cells.

Seasonal Variation of the Crop Jsc

The present experiments have indicated that in addition to storing food and producing crop "milk" the pigeon crop plays a role in salt transport. We have noticed that the transport rate *in vitro* has a seasonal variation (Table 10). The Jsc values are highest in the spring and summer, which is the most active time of breeding according to Levi (1957). This correlation may indicate that the crop serves to replenish salt lost in egg and crop "milk" production (which both male and female pigeons use to feed the young).

Control of Seasonal Variations of Jsc

Attempts to determine what factors *in situ* may regulate the transport rate have not been successful. Although changes in osmolarity or in concentrations of Ca^{++} or Cu^{++} have been shown to affect the crop Jsc, it seems unlikely that any of these factors *in situ* have seasonal variations large enough to regulate salt absorption.

Transport processes in other animal tissues have been shown to be regulated by hormones. No hormone treatment on the crop has produced a statistically significant effect on the transport mechanism. High doses of lentin did increase the measured *in vivo* Jsc (Figure 18), but the action of the drug may have been on the blood supply to the tissue rather than directly on the

transport mechanism. The application of pitressin, epinephrine, prolactin and aldosterone did not have measurable effects on the Jsc or PD of the crop. This indicates that the crop transport mechanism has different controls from other ion transport systems.

Model of Ion Transport in the Crop Epithelium

A model of crop epithelial ion transport is presented on the basis of information derived from this study and from the study by Rose (1967). Figure 20 schematically represents the cell layers of the s. disjunctum, s. spinosum and s. basale seen in Figure 19 and in other (unpublished) photo micrographs of the crop tissue. Previous experiments *in vitro* by Rose (1967) have shown that ^{22}Na and ^{36}Cl reach a seven times larger space when bathing the membrane on the serosal side as opposed to the mucosal side. Therefore, the main permeability barrier to these ions is probably located close to the mucosal surface, perhaps near the junction of the s. disjunctum and s. spinosum.

Evidence that the permeability barrier is located on the mucosal side of the transport cells comes from pH studies (p. 61). The Na^+ transport rate (Jsc) is unaffected by extreme H^+ concentrations in the mucosal solution but is rapidly reduced by H^+ concentrations outside the pH range 7.4-7.7 at the serosal surface. Assuming that the main permeability barrier to H^+ is the

Figure 19.--Photomicrograph of the crop cross-section (x 4400). Layers of epithelium from mucosa (left) to basement membrane (right): SD, stratum disjunctum; SS, stratum spinosum; SB, stratum basale. The proportions and position of structures in Figure 20 are derived from this photomicrograph.

Figure 20.--Model of ion transport in the crop epithelium. Na^+ diffuses from the mucosal solution through extracellular paths of the stratum disjunctum (SD) and through the main permeability barrier (PB) of the membrane into the transport cells of the stratum spinosum (SS) and stratum basale (SB). Na^+ is then actively transported toward the serosal surface in exchange for K^+ which is transported into the cells. Dotted lines represent diffusion paths. Intracellular K^+ will tend to diffuse down its concentration gradient back to the serosal solution. Cl^- crosses the membrane passively from mucosa to serosa in response to the electrical gradient established by the active cation transport.

mucosal surface
SD
SS
SB
PB
Na^+
K^+
Na^+
K^+
Na^+
serosal surface

same as that which restricts Na^+ and Cl^-, then this barrier must be located on the mucosal side of the transport cells.

Hyperosmotic solutions at the mucosal surface decrease the membrane R and hyposmotic solutions increase the R. Since the most likely site of osmotic action is on the cells bordering the lumen, we can assign some of the membrane resistance to the cells of the s. disjunctum. A possible mechanism of action of the hyperosmotic and hyposmotic solutions is by their effect on cell volume which regulated the volume of the extracellular spaces. The increase in Jsc in response to hyperosmotic solutions bathing the mucosal surface may be the result of more Na^+ reaching the transport cells through the s. disjunctum because of larger extracellular pathways.

Na^+ is probably exchanged at the pump site for K^+ from the serosal solution since a depletion of K^+ from the serosal solution results in a lower Jsc (Figure 16). Ion exchange at the pump site is an energy requiring process as evidenced by the decreased Jsc in response to metabolic inhibitors used by Rose (1967).

CHAPTER VI

SUMMARY

A study of the potential difference (PD), short-circuit current (Jsc) and R of the pigeon crop membrane has been made under *in vivo* and *in vitro* conditions. The results of this study indicate that there is an active absorption of Na^+ by the crop *in vivo*.

The Jsc both *in vivo* and *in vitro* is reduced when the mucosal surface of the crop is bathed with a Ringer solution which has the Na^+ concentration reduced by either choline substitution or by water dilution. The R increased both *in vivo* and *in vitro* in response to the water diluted (hyposmotic) Ringer solution but not in response to the choline substituted (isomotic) Ringer. The R increase may be due to a decrease in the membrane permeability to ions resulting from swelling of the tissue cells and a consequent closure of extracellular spaces.

Ringer solutions bathing the mucosal surface which were made hyperosmotic with sucrose, ethanol or DMSO resulted in an increase in the Jsc and a decrease in the R. This osmotic effect may be an opening of the

extracellular paths to ions which allows Na^+ an easier entry form the mucosal solution to the transport cells. The effects of ethanol and DMSO on the crop resistance are not greater than the effect of sucrose at the same osmotic concentration, indicating that these molecules (ethanol and DMSO) have no specific chemical action on the permeability characteristics.

The average resistance of the crop membrane in vivo is lower than in vitro. The permeability characteristics of this tissue change in response to in vitro conditions, as do those of frog skin according to Brown (1962). Results from these two tissues indicate that care should be taken when applying results from in vitro experiments to whole animals.

When a Ringer solution made with $SO_4^=$ rather than Cl^- as the main anion, bathed the mucosal surface of the crop membrane in vivo or in vitro there was an increase in resistance. This is the expected result if the membrane is less permeable to $SO_4^=$ than to Cl^- because there will be less conductivity in the membrane.

$CuSO_4$ ($6X10^{-4}M$) in the Ringer solution bathing the mucosal surface of the crop in vivo or in vitro resulted in an increase in the Jsc but no change in resistance. This is an unexplained difference from the effect on frog skin where $CuSO_4$ increased the resistance and PD but did not affect the Jsc.

A seasonal variation in the rate of Na^+ absorption by the crop *in vitro* has been noticed but efforts to control the rate with hormones have not been successful.

The presence of Ca^{++} (4.3 mEq/l) in the Ringer solution *in vivo* or *in vitro* is associated with a lower Jsc and higher resistance than when a Ca^{++} free Ringer solution is used. Ca^{++} may restrict the entry of Na^+ from the mucosal solution to the Na^+ transport cells.

The Jsc is reduced when Li^+ is substituted for Na^+ in both the mucosal and serosal solutions *in vitro*, indicating that Li^+ can not substitute for Na^+ on a one for one basis in the transport mechanism. When K^+ of the serosal solution is replaced by Cs^+ or Rb^+ the Jsc is reduced, indicating that these ions can not substitute for K^+ in the transport mechanism.

A model of ion transport in the crop epithelium is presented on the basis of the results of this study and the previous study by Rose (1967).

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APPENDICES

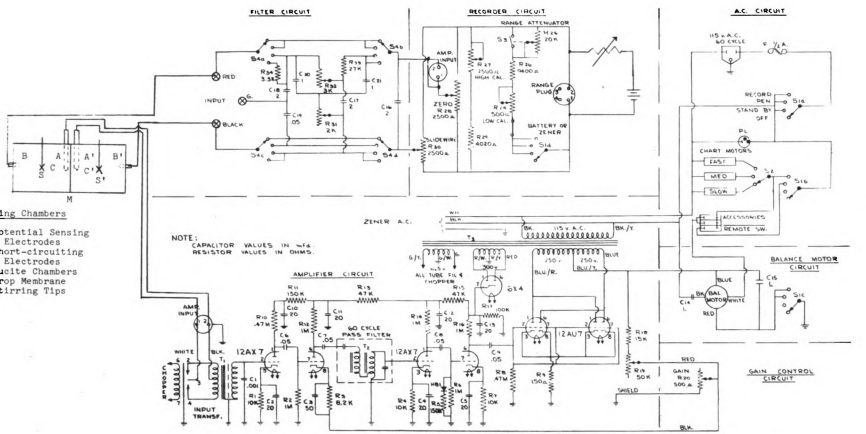
APPENDIX I

SOURCE OF CHEMICALS, DRUGS AND HORMONES

Reagent	Source
CsCl	E. H. Sargent and Co., Detroit, Mich.
LiCl	Fisher Scientific Co., Fair Lawn, N.J.
RbCl	E. H. Sargent and Co., Detroit, Mich.
^{22}Na	Abbott Laboratories, Chicago.
agar	E. H. Sargent and Co., Detroit, Mich.
fetal calf serum	Grand Island Biological Inc., Grand Island, N.Y.
tris buffer	Fisher Scientific Co., Fair Lawn, N.J.
dl-leucine	Merck and Co., Rahway, N.J.
dl-alanine	Eastman Kodak Co., Rochester, N.Y.
Na Pentobarbital	Sherman Drug and Chemical Co., New York, N.Y.
dimethyl sulfoxide (DMSO)	Mann Research Laboratories, New York, N.Y.
aldosterone	Calbiochem, Los Angeles.
epinephrine	Mann Research Laboratories, New York.
lentin (carbachol chloride)	Merck and Co., Rahway, N.J.
pitressin	Parke Davis and Co., Detroit, Mich.
prolactin PB-1	NIH Endocrine Study Section, Bethesda, Md. (donated)

APPENDIX II

MODIFIED SARGENT RECORDER MODEL SR



Bathing Chambers

A,A'--Potential Sensing Electrodes
B,B'--Short-circuiting Electrodes
C,C'--Lucite Chambers
M --Crop Membrane
S,S'--Stirring Tips

NOTE: CAPACITOR VALUES IN mfd. RESISTOR VALUES IN OHMS.

SARGENT RECORDER MODEL SR SCHEMATIC

APPENDIX III

PROCEDURE FOR COUNTING ^{22}Na

Radioactive samples were counted in 15 ml of a solution made from the following recipe:

80 g Naphthalene
5 g PPO (2,5-Diphenyloxazole)
50 mg Alpha-NPO (2-(1-Naphthyl)-5-Phenyloxazole
Add dioxane to one liter

Counting solutions were used within three months following mixing.

Samples were counted on a Nuclear Chicago Mark I liquid scintillation spectrometer. The amount of quenching was found by the method of quench correction described in the instruction manual of the Mark I using a ^{133}Ba external standard. The amount of quenching in the individual sample vials was approximately the same and could be ignored.