By salt depleting the animals or by injecting them with aldosterone, I was able to increase the active transport of sodium across the isolated skin. The potential difference was increased by 37% and 43% respectively and the net sodium flux by 69% and 62%. Exposure of frogs to 50 mM NaCl did not produce a significant change in the rate of sodium transport. Although the resistance of the skins did not show a significant change as a result of the treatments, they did show trends. The skins from salt-depleted and aldosterone injected frogs are less resistant than the controls. There was no correlation between the cholinesterase activity in the skin and the rate of active transport of sodium across the skin.
CHOLINESTERASE ACTIVITY AND SODIUM TRANSPORT IN THE FROG SKIN

by

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Typed by Ruth Baines
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I wish to express my appreciation for the guidance, assistance, kindness and patience given to me by Professor R. Alvarado.
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CHOLINESTERASE ACTIVITY AND SODIUM TRANSPORT IN THE
FROG SKIN

INTRODUCTION

Substances such as inorganic ions, water, amino acids and sugars are constantly being transferred across the membranes of living cells. C. R. Park \((49)\) states that at least nine different mechanisms, including simple diffusion, solvent drag, facilitated diffusion, exchange diffusion, active transport, phagocytosis and pinocytosis may be involved in this activity. I shall be concerned only with active transport and in particular with the way in which the rate of active sodium transport varies with the activity of the enzyme cholinesterase.

Linderholm \((41)\) has defined active transport as that process which makes possible a transfer of substances across a cell membrane from a lower to a higher electrochemical potential and which depends upon metabolically maintained enzymatic reactions located in or at the cell membranes. The ability to actively transport substances across membranes enables the cell to accumulate from its environment substances needed to sustain life, to excrete waste products or toxic substances, to maintain potentials across membranes and to maintain a constant cell volume.
The isolated frog skin has been the source of much of the information which is now known about the active transport of sodium across a membrane. Attention was drawn to it in 1848, when Du Bois Reymond (61) reported that an isolated piece of frog skin maintains a potential difference between the outside and the inside solutions, the inside being positive to the outside. In 1904, Galeotti (61) showed that sodium was essential for the maintenance of this potential difference.

In 1949, Ussing (59), using radioactive sodium showed that even when the experimental conditions were varied by changing the concentration of the sodium in the solution bathing the outside of the skin or the pH of the solution, the amount of sodium passing across the membrane from the outside solution to the inside solution (sodium influx) is always greater than the amount of sodium passing to the outside solution from the inside solution (sodium efflux). This means that sodium is being concentrated in the solution bathing the inside of the skin.

Krogh had shown in 1938 (62) that intact salt-depleted frogs would take up both sodium and chloride ions when placed in a very dilute salt solution. For this reason Ussing (59, 61) studied the movement of chloride ion in addition to that of sodium in order to determine whether both ions are subject to active transport across
the isolated skin.

In order to make this distinction Ussing derived and applied the "flux equation" which states that for an ion that moves through the membrane passively, the flux ratio, which is equal to the inward flux divided by the outward flux, can be expressed by the equation

\[
\frac{M_i}{M_o} = \frac{C_o}{C_i} \cdot \frac{f_o}{f_i} \cdot \frac{zF}{RT} \exp\left(\frac{\psi_o - \psi_i}{RT}\right)
\]  

where \( M_i \) = influx

\( M_o \) = efflux

\( C_o \) = concentration of the ion in the outside solution

\( C_i \) = concentration of the ion in the inside solution

\( f_o \) and \( f_i \) = activity coefficients of the ion on each side of the membrane

\( z \) = charge on the ion

\( F \) = Faraday

\( R \) = gas constant

\( T \) = absolute temperature

\( (\psi_o - \psi_i) \) = potential difference = \( E \)

If the activity coefficients are equal on each side of the membrane, the equation for a univalent ion becomes:

\[
\frac{M_i}{M_o} = \frac{C_o}{C_i} \cdot \frac{EF}{RT} \exp\left(\frac{\psi_o - \psi_i}{RT}\right)
\]
All of the terms in this equation can be evaluated for a specific ion. Any deviations of the flux ratio from the value predicted by the equation would mean that that particular ion was moving across the membrane by some process other than passive diffusion. When sodium and chloride fluxes were determined, it was found that the flux ratio for sodium was much larger than predicted by the flux equation, whereas the ratio for chloride was as predicted. Ussing concluded that chloride ion moves inward passively following the potential gradient across the skin and acts as a partial shunt of the skin potential.

In 1951 Ussing and Zerahm (64) showed more conclusively that only sodium is actively transported across the isolated frog skin. They short-circuited the skin by placing it between two chambers containing identical Ringer's solutions and then adjusting an externally applied voltage so that the potential difference across the skin was zero. Since both the chemical and electrical potential are abolished, any ions which move across the skin must do so by means of active transport and the current so generated can be measured by a microammeter. By using two radioactive isotopes of the same ion, the simultaneous efflux and influx could be obtained. Using Na$^{22}$ + Na$^{24}$, Ussing and Zerahm (64) found that only sodium is actively transported across the isolated frog skin and
that the net inward flux of sodium, \( M_{\text{net}} = M_1 - M_c \) as determined by isotope, accounted for the current generated across the skin as measured with a microammeter. When the chloride fluxes were measured in the same way using \( \text{Cl}^{36} \) and \( \text{Cl}^{38} \) (33), the inward chloride flux was found to be exactly equal to the outward chloride flux, thus confirming that the chloride ion moves across the membrane passively.

Once it was established that sodium is transported across the isolated frog skin against an electrochemical gradient, experiments were and still are being carried out to determine the cellular mechanism involved. To gain insight into the process, attempts have been made to determine the specificity of the transport, where in the cell or skin the sodium pump is located, the energy requirements (or in other words, the relationship of transport to the metabolism of the cell), and what enzymes are involved in the transport.

When various cations were substituted for sodium in the solution bathing the outside of the frog skin, only lithium replaces sodium in maintaining the potential difference (70). However, lithium is not transported across the skin as efficiently as sodium and accumulates in the skin, eventually inhibiting the transport process.

Potassium proved to be essential to the transport process. If potassium is left out of the solution bathing the inside of the skin,
the potential difference disappears and if the concentration of potassium in the inside solution is either too high or too low, the potential difference drops (61, 27).

Since, by definition, energy is assumed to be required when active transport takes place, one would expect that the metabolic rate or oxygen consumption would vary with the amount of active transport taking place, that oxygen is probably essential for transport, and that ATP is involved. That oxygen is required for maximum transport was shown by Lund (43) as early as 1926 and by Francis, Pumphrey and Gatty (19, 21, 20) in the 1930's. They noticed that the skin potential decreased during oxygen starvation, that the potential difference was dependent upon the oxygen concentration, that the respiratory inhibitors cyanide, iodoacetate and carbon monoxide also caused a decrease in the potential difference across the skin and that endogenous carbohydrates and exogenously applied dl-lactate, pyruvate, acetate, propionate, n-butyrate and isobutyrate support the potential. Zerahn (69) using the frog skin, and Leaf and Renshaw (39) using the toad bladder, showed that oxygen consumption increases during active transport. Skou (57, 58) and Post and Albright (51) found a very close connection between Na-K ATPase activity and active transport in red blood cells.

There is evidence that Na-K activated ATPase is present in the
toad bladder (3) and in the frog skin (17).

Experiments which gave some indication as to where in the frog skin the pump is located were reported by Koefoed-Johnsen and Ussing in 1958 (37). They found that when the chloride shunt is removed by replacing the chloride ion in the solutions by the very slowly penetrating sulfate ion and the concentration of sodium in the outside solution is varied, the skin behaves like a sodium electrode. When the potassium concentration in the inside solution is varied, the skin behaves like a potassium electrode. From these results, they concluded 1- that the outer membrane of the skin is permeable to sodium and chloride, but relatively impermeable to potassium, 2- that the inner membrane is permeable to potassium and chloride, but only slightly permeable to sodium and sulfate, 3- that the sodium pump is located on the inner boundary of the skin.

Koefoed-Johnsen and Ussing (1958) then proposed the "two membrane theory" of active transport (37). According to the theory, sodium diffuses through the outer, sodium-permeable membrane from the outer solution into the cell. The sodium then moves, or is carried, to the opposite side of the cell where it is pumped across the inner, potassium-permeable membrane in exchange for potassium. The K\(^+\) then diffuses back into the inner solution because its concentration in the cell is higher than in the inner solution. The
sodium pump, therefore, maintains a low cellular concentration of sodium and a high cellular concentration of potassium. The potential difference across the frog skin is equal to the sum of the sodium diffusion potential \( (E_o) \) of the outer membrane and the potassium diffusion potential \( (E_i) \) of the inner membrane:

\[
E = E_o + E_i = \frac{RT}{F} \ln \frac{[Na^+]_0}{[Na^+]_{cell}} + \frac{RT}{F} \ln \frac{[K^+]_{cell}}{[K^+]_i}
\]  

(3)

Since the transport of sodium would be linked to potassium in a 1:1 relationship, the pump itself would not be responsible for the development of the potential and may be considered non-electrogenic. However, some recent experiments indicate that the sodium pump may be electrogenic and that the sodium-potassium exchange is not a 1:1 exchange (5, 6, 8, 16).

To test the idea that the pump is located in the inner membrane, the electrical profile of the skin was determined by puncturing the skin with micro-electrodes. One would expect one or two potential jumps. Ottoson (48) found that the main part of the resting potential occurred across a submicroscopic membrane between the epidermis and the corium. Later studies by Engaek and Hoshiko (15) showed that the potential difference was formed in two successive steps, the first occurring in the epidermis and the second in the junction between the epidermis and the corium. Sheer and Mumbach (53) also found two distinct jumps, the first located in the stratum germinativum which is the lower layer of the epidermis and the second in the tela subcutanea which is below the dermis of the skin. More recently, Ussing and Windhager (63) indicated that a discontinuous electrical
potential existed within the epithelium and that the first jump occurs just beneath the cornified layer and others occurred in steps down to the basement membrane, which is located between the epidermis and the dermis. This finding would not necessarily invalidate the two membrane theory.

A considerable amount of additional information has been obtained by studying the effects of hormones and enzyme inhibitors on active transport. The hormones adrenalin, vasopressin and aldosterone all increase the rate of sodium transport, probably by controlling the amount of sodium entering the skin from the outside solution. This would increase the sodium pool in the skin and there is evidence that the amount of sodium reaching the pump is the major limiting factor in determining the influx of sodium (1, 1, 11, 12 23, 23, 38, 44, 46, 47, 50, 56, 59). Animals, in which sodium transport was stimulated as much as possible with aldosterone, still show an increase in rate of transport when treated with vasopressin. Evidently the two hormones affect active transport in different ways (12).

Several enzymes have been implicated in the transport process. As mentioned above, a sodium-potassium activated adenosine triphosphatase has been demonstrated in several membranes
which transport sodium, including the frog skin (17) and the toad bladder (3). Cholinesterase is the other main enzyme which has been associated with active transport and whose connection with sodium transport has been debated for many years.

Van der Kloot (65, 66) demonstrated that when the anticholinesterases, hexaethyltetrathosphate or physostigmine (eserine) were added to preparations of the sartorius muscle of the frog the extrusion of sodium from the muscle and the cholinesterase activity decreased proportionately. The degree of inhibition of both activities was dependent upon the concentration of the inhibitors. Van der Kloot concluded that cholinesterase activity might be correlated with the active transport of sodium.

A similar decrease in active transport by cholinesterase inhibitors has been observed in red blood cells (25, 26) in the isolated gills of the crab Eriocheir sinensis (36), and in the crayfish kidney (29, 30).

Kirschner (31) found that the cholinesterase inhibitors, tetraethylpyrophosphate and physostigmine inhibited sodium transport when added to the solution bathing the inside of an isolated frog skin. The inhibition was greatly reduced if they were added
to the outside solution. In 1958 Koblick demonstrated that cholinesterase is present in the frog skin, most of it being concentrated in the tela subcutanea (33). These results seem to implicate cholinesterase in the transport of sodium across the skin.

Further evidence that cholinesterase might be involved was given by Fleming (18), who showed that when the tela subcutanea, which contains most of the cholinesterase activity, is removed, the skin no longer actively transports sodium and by Koblick, Goldman and Pace (35), who by comparing the short-circuit currents of isolated frog skins with their corresponding cholinesterase activities, showed that the magnitude of the current varied with the cholinesterase activity.

Evidence that cholinesterase may not be directly involved in the active transport of sodium is given by the work of Franz and van Bruggen (22) who showed that the sodium pump in the frog skin is still functional after the tela subcutanea is removed, indicating that the pump is in the epidermis and not in the tela subcutanea where over 90% of the skin cholinesterase is located. Additional evidence that cholinesterase activity and active transport are not directly correlated comes from Pratley (52) who showed that the cholinesterase activity decreased with age while the electric power (EI) increased with age. He argues that the enzymes associated
with the production of the electric power should vary directly as the product EI.

I propose to experimentally alter the rate of active sodium transport across the skin of live frogs and determine if there are corresponding changes in cholinesterase activity in the skin. Jorgensen has shown that when frogs are kept in distilled water (salt depleted) for a few days, the rate of active transport is augmented (28). The increase in sodium transport is accompanied by and probably results from an increased secretion of aldosterone (2, 13). The rate of active transport can also be increased by injecting frogs with aldosterone (1, 11, 12, 49). The rate of transport and the secretion of aldosterone can be decreased by placing the frogs in a concentrated saline solution (47, 59).

The frogs, after being treated by the methods indicated above, will be sacrificed and the skin from the ventral body surface will be tested for cholinesterase activity, potential difference, and short-circuit current.
METHODS

The frogs, Rana pipiens, after being received from Wisconsin, were kept in a shallow fiber-glass lined metal trough partially filled with dechlorinated tap water at 18°C. The water was changed every four or five days.

For treatment the frogs were divided into four groups of about twelve animals each. They were kept in rectangular plastic containers filled with three liters of solution with four frogs to a container.

1. Group I consisted of the control frogs which were kept in a 1mM sodium chloride solution.

2. Group II consisted of salt-depleted frogs. The frogs were salt depleted by placing them in three liters of distilled water with additional distilled water flowing through the container at the rate of about six liters per day. The frogs were treated for at least nine days before being sacrificed.

3. Group III consisted of salt-loaded frogs which were treated by being placed in three liters of 50mM sodium chloride for at least one week.

4. Group IV consisted of aldosterone treated animals. Ten micrograms of aldosterone was injected into the dorsal lymph sac of these animals each day for 6-10 days. The aldosterone
solution was prepared by dissolving one milligram aldosterone in 1 ml 95% ethanol and diluting it to 10 ml with frog Ringer's solution. Control frogs were similarly injected with an equivalent volume of the diluant.

After treatment, the frogs were sacrificed by double-pithing. The ventral abdominal skin was removed. The upper section was placed in a barbitol buffer solution and was used for the determination of cholinesterase activity. The remaining portion was placed in frog Ringer's until it could be mounted in the chambers.

The skin in the Ringer's solution was mounted in the short-circuit chamber (see Fig. 1 for specifications). About 15 milliliters of Ringer's solution was added to each side of the skin and the initial potential difference and short-current were measured as described by Ussing and Zerahn (64). Currents corresponding to voltages of ten to sixty millivolts applied to the outside of the skin were then measured so that the resting current, potential difference and resistance could be determined (see results). The potential difference and current were measured at intervals up to thirty minutes for about two and one half hours after the skin was mounted.

In order to verify that, in spite of the treatments, the current generated by the short-circuited skin is an indication of the influx of sodium, the influx of sodium across some of the skins was
C: Chamber (1.04 cm dia) containing, on each side of skin, about 15 ml. Frog Ringer's.
S: Skin.
A, A': Agar-Ringer bridges, connecting outside and inside solutions, respectively, with calomel electrodes.
B, B': Agar-Ringer bridges used for applying outside E.M.F.
D: Battery.
P: Potentiometer.
M: Microammeter.
E: Saturated KCl solution.
a: Inlets for air.

Figure 1. Diagram of apparatus used for determining P. D. and short-circuit current.
measured with Na$_{22}$. The isotope (about 0.5 microcuries) was added to the outside solution. Two milliliter samples taken from the inside solution at one-half hour intervals for two hours were placed in planchets and evaporated to dryness. The samples taken were replaced by equal quantities of frog Ringer's solution. At the end of two hours a sample of the outside solution was assayed for radioactivity using a gas flow counter, while another sample was analyzed for sodium by flame photometry. From this the specific activity of the outer solution was computed. The influx was calculated from the rate of appearance of Na$_{22}$ in the inner solution and from the specific activity of the outer solution with appropriate corrections for the sample.

The equation for the influx is

$$M_i = \frac{dc}{S.A.} dt$$

(4)

where: $M_i = $ sodium influx

$$\frac{dc}{dt} = $ rate of appearance of isotope in the inner solution

S. A. = specific activity

It is assumed that the backflow is negligible (64) and that the specific activity in the outer solution remains constant.

As soon as the posterior part of the skin was mounted in the short-circuit chamber, the anterior part of the skin was prepared for the cholinesterase determination. The method used was
Koblick's (33) modification of Hestrin's (2) hydroxamate method.

The skin was divided into three approximately equal sections, one section being used as a tissue blank and the other two being used to determine cholinesterase activity. Each section was blotted and placed in a glass-stoppered flask containing 0.5 milliliters of barbitol buffer. A reagent blank, two substrate standards and a known cholinesterase solution were prepared at the same time.

All the flasks were placed on a shaker which suspended the flasks in a water bath, the temperature of which was kept at 37°C. The contents of the flasks were allowed to equilibrate for about 15 minutes. Then 0.5 milliliters of the acetylcholine chloride substrate (final concentration in the flask $5.5 \times 10^{-3}$ M) was added to all the flasks except the reagent blank and the tissue blank. To these flasks 0.5 ml of buffer was added. The flasks were then stoppered and agitated in the water bath for two hours.

*Immediately after two hours 0.5 ml was taken from each flask and added to tubes.*

*The original method specified that the samples be incubated for one hour instead of two. Although I used equivalent amounts of tissue, I was not able to detect any cholinesterase activity if the samples were incubated for a period less than one and one-half hours. A standard curve for acetylcholine was made by using graded concentrations of acetylcholine chloride. The relationship between concentration and optical density is linear over the concentration range $0.55 \times 10^{-3}$ to $5.5 \times 10^{-3}$ M/liter. All enzyme activity was abolished when the skins were incubated with the cholinesterase inhibitor, eserine sulfate ($6 \times 10^{-5}$ M), for thirty minutes before adding the acetylcholine substrate.
containing 0.5 ml of alkaline hydroxylamine solution. Each tube was shaken. The color was developed by adding 4 ml of the FeCl₃ solution to each tube and the contents mixed. The optical density of each tube was determined using a Klett Colorimeter with a #54 filter. The pieces of skin were removed from the flasks, blotted and weighed. They were then dried and reweighed.
Reagents:

1. Frog-Ringers

   \[
   \begin{align*}
   &\text{NaCl} & 26.8 \text{ grams} \\
   &\text{KCl} & 1.184 \text{ grams} \\
   &\text{CaCl}_2 \cdot 2\text{H}_2\text{O} & 0.60 \text{ grams} \\
   &\text{NaHCO}_3 & 1.68 \text{ grams} \\
   
   \end{align*}
   \]

   The above were dissolved in 4 liters of distilled water.

2. Barbitol Buffer

   Sodium diethylbarbiturate & 2.06 \text{ grams} \\
   Anhydrous Na$_2$CO$_3$ & 2.12 \text{ grams} \\

   Dissolve the above in 180 ml distilled water.

   Add

   About 1.9 ml concentrated HCl

   MgSO$_4$ \cdot 7\text{H}_2\text{O} & 2.18 \text{ grams} \\
   KCl & 0.04 \text{ grams} \\

   Dilute to 200 ml with distilled water and adjust the pH to 8.

   Refrigerate.

3. Hydroxylamine 14%

   NH$_2$OH, HCl & 14.0 \text{ grams} \\

   Dilute to 100 ml. Refrigerate.

4. NaOH 28%

   NaOH & 28.0 \text{ grams} \\

   Dilute to 100 ml.
5. Alkaline hydroxylamine

Mix equal volumes of 3 and 4 just before use.

6. FeCl₃ Reagent

FeCl₃·6H₂O 2.25 grams

Concentrated HCl 3.6 ml

Dilute to 100 ml with distilled water.

7. Stock Acetylcholine Solution \((11 \times 10^{-3} \text{ M})\)

Acetylcholine 0.1 grams

(Merck, 100 mg ampules)

Dissolve in 50 ml of barbitol buffer. Refrigerate.

8. Acetyl cholinesterase from Bovine erythrocytes, Type 1,
Sigma Chemical Company.
RESULTS

The results are summarized in Table 1 and Figure 3.

The net flux of sodium was determined using the equation (7)

\[ M_{\text{net}} = \frac{It}{F} \]  \hspace{1cm} (5)

\( M_{\text{net}} \) = net flux of Na⁺

\( I \) = short-circuit current in microamperes during a specified time period

\( t \) = time in seconds (3600 seconds)

\( F \) = Faraday constant = 96,494 coulombs/mole

The short-circuit value taken at one and one-half hours after the frog was pithed was used in all cases.

The resistance in ohm.cm² of each skin was calculated using the equation (46)

\[ R = \frac{-E}{I} \]  \hspace{1cm} (6)

\( R \) = resistance in ohm.cm²

\( E \) = potential difference in microvolts

\( I \) = current in microamperes/cm²

The values for E/I were obtained by plotting the values found for the current at specified voltages which were applied to the skin. This gives a straight line, a representative curve is shown in Figure 2.
Table 1. Summary of the effects of various treatments on sodium transport and cholinesterase activity. Values represent mean ± standard error.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>P. D. (mV.)</th>
<th>Cholinesterase activity</th>
<th>M_{net} (µeq/hr/cm²)</th>
<th>Resistance (ohm cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>23.1 ± 2.8</td>
<td>14.1 ± 0.7</td>
<td>1.11 ± 0.15</td>
<td>785 ± 44†</td>
</tr>
<tr>
<td>Aldosterone control</td>
<td>10</td>
<td>24.1 ± 3.7</td>
<td>12.2 ± 0.9</td>
<td>1.26 ± 0.20</td>
<td>751 ± 57</td>
</tr>
<tr>
<td>Salt depleted</td>
<td>13</td>
<td>32.7 ± 3.2*</td>
<td>14.9 ± 0.8</td>
<td>1.87 ± 0.17**</td>
<td>625 ± 50</td>
</tr>
<tr>
<td>Salt loaded</td>
<td>11</td>
<td>20.0 ± 3.5</td>
<td>12.9 ± 0.8</td>
<td>1.10 ± 0.19</td>
<td>806 ± 54</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>10</td>
<td>33.2 ± 3.7*</td>
<td>13.7 ± 0.9</td>
<td>1.80 ± 0.20**</td>
<td>688 ± 57</td>
</tr>
</tbody>
</table>

* - Significant at the 5% level with respect to the control.
** - Significant at the 1% level with respect to the control.
† - Based on 17 observations.
1 - Micromoles of acetylcholine chloride hydrolyzed in 2 hours per gram of wet skin.
2 - Net flux of sodium calculated from the short-circuit current.
Figure 2. Regression line of current (I) on Potential (E) across Frog skin #4.
Figure 3. Summary of the effects of treatment on active transport and cholinesterase activity.
Effect of the Treatments on the Sodium Transport

Control frogs:

The potential difference across the skins of the control frogs has a mean value of $23.1 \pm 2.8$ millivolts, with values ranging from 3.0 to 54.0 millivolts. These values are within the range of values reported by other investigators (61). The net sodium flux for the control skins averaged $1.11 \pm 0.15$ microequivalents/hour/centimeter squared. The mean of the resistances is $785 \pm 44$ ohm-cm$^2$. The last two means are also within the range of values reported by other investigators (1, 64). The potential difference, currents and resistances of the individual frogs varied considerably and the frogs which had been kept in the laboratory for any length of time without food tended to give lower values. This was accounted for in the design of the experiments. The mean cholinesterase activity in the control skins is $14.1 \pm 0.6$ gr./2 hrs with values ranging from 9.4 to 22.2. The difference between duplicate runs of parts of the same skin did not exceed 8.1 and were usually within 3% of each other. These values correspond well with those obtained by Koblick (34, 35) using the same method.
Salt Depletion

Salt depletion stimulates active sodium transport across the frog skin. This is reflected by a 43% increase in the potential difference relative to the controls and more importantly by a 68% increase in the short-circuit current. Both differences are significant. The mean resistance of the skin dropped to 80% of the control value. The cholinesterase values were not significantly different from those of the controls.

Salt Loading

The animals which were salt loaded did not show a statistically significant decrease in the rate of active transport. The average values for the potential difference and the calculated net sodium flux were slightly lower than the average control values. The mean resistance of the skins was slightly higher than that of the control and there was no change in the cholinesterase activity. The animals were salt loaded in a 50mM NaCl solution which was probably not concentrated enough to effectively inhibit the active transport mechanism. A 100mM or a 125mM NaCl solution would probably have been more effective.
Aldosterone Treatment

The animals which were injected with aldosterone showed a definite augmentation in the transport of sodium across the skin. The increase in potential difference over the control values was significant at the 2.5% level and the current and the calculated sodium flux were significant at the 0.5% level. There was a 62% increase over the control in the amount of sodium transported across the skin. The mean resistance dropped to 86.5% of the control value. Again, the cholinesterase activity did not change. The transport of the control aldosterone animals was not statistically different from that of the control animals, so evidently the small amount of ethanol in the injected solution and the handling of the frogs necessary for making the injections did not have any effect on the active transport.

Although the active transport was increased by salt depleting the frogs or by injecting aldosterone, the cholinesterase activity did not show a corresponding change.

The values for the sodium influx obtained using radioactive sodium were definitely higher than those calculated from the current (Table 2). Ordinarily, these values are within 5% of each other, the influx being a little higher than the net flux due to a small outflux of sodium (64).
Table 2. Sodium influx and net sodium flux for individual skins.

<table>
<thead>
<tr>
<th>Skin #</th>
<th>$M_i$ (μeq/hr/cm$^2$)</th>
<th>$M_{net}$ (μeq/hr/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>3.06</td>
<td>1.95</td>
</tr>
<tr>
<td>Aldosterone control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>2.28</td>
<td>1.45</td>
</tr>
<tr>
<td>34</td>
<td>1.64</td>
<td>0.81</td>
</tr>
<tr>
<td>36</td>
<td>2.94</td>
<td>1.88</td>
</tr>
<tr>
<td>44</td>
<td>2.58</td>
<td>2.00</td>
</tr>
<tr>
<td>Salt depleted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>3.06</td>
<td>1.96</td>
</tr>
<tr>
<td>Salt loaded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>1.53</td>
<td>0.79</td>
</tr>
<tr>
<td>37</td>
<td>1.65</td>
<td>0.88</td>
</tr>
<tr>
<td>38</td>
<td>2.00</td>
<td>1.52</td>
</tr>
<tr>
<td>Aldosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>4.29</td>
<td>2.67</td>
</tr>
<tr>
<td>32</td>
<td>2.30</td>
<td>1.81</td>
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<tr>
<td>35</td>
<td>2.35</td>
<td>1.91</td>
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<tr>
<td>41</td>
<td>1.41</td>
<td>1.09</td>
</tr>
<tr>
<td>43</td>
<td>2.35</td>
<td>1.76</td>
</tr>
</tbody>
</table>

$M_i$ = sodium influx determined by using Na$^{22}$.  
$M_{net}$ = net sodium flux calculated from the short-circuit current.
DISCUSSION

According to the model for active transport proposed by Koefoed-Johnsen and Ussing (37), the sodium ions, after passively entering the cell through the sodium-permeable membrane, pass to the opposite side of the cell where they are pumped out into the inside solution against an electrochemical gradient. The means by which the sodium is actively transported across the membrane is still not clear. The carrier hypothesis is the most favored of the mechanisms proposed. In 1955 Kirschner (32) formulated a model for the carrier-mediated transport of sodium across the frog skin. He assumed that the sodium forms a non-ionized complex with the carrier at the inner surface of the portion of the cell membrane facing the inner solution. The complex migrates across the cell membrane and releases sodium ion into the inside bathing solution. Enzymes are most likely associated with the process and in 1959 Koblick (33) proposed an enzymatic ion exchange model for active sodium transport. He assumed that the membrane bears pores and that the walls of some of these pores contain the active surface of the enzyme cholinesterase. The active surface of cholinesterase has anionic sites which attract the sodium ion. When the substrate, in this case acetylcholine, diffuses into the pore it combines with the enzyme to form a transient enzyme-substrate complex which
decreases the anionic field strength of the enzyme displacing the sodium ion. The acetylcholine is hydrolyzed and the anionic site is free to attract another sodium ion.

The reason for involving cholinesterase in sodium transport comes from experiments which have shown that anticholinesterases depress transport. Since these inhibitors might be affecting the transport in ways not involving cholinesterase, Koblick, Goldman and Pace (35) showed that there was a correlation between the amount of cholinesterase activity in the skin and the active transport of sodium across the skins of randomly picked frogs. While this seems to implicate cholinesterase in the transport mechanism, it is not unequivocal evidence, since in a population of frogs it is possible that both cholinesterase activity and sodium transport are affected by some other factor(s) and that the correlation is fortuitous.

Another approach not involving enzyme inhibitors, is to experimentally alter the sodium transport and then determine the cholinesterase activities. When this was done, no significant change in the cholinesterase activities was observed, even when the rate of sodium transport was increased by 68%. Therefore, a change in the active transport does not mean that a change in the cholinesterase activity has also occurred. This conclusion does not support Koblick's ion exchange model (34) nor the conclusions arrived at by Kirschner and Fleming (31, 13). But it does support the findings
of Franz and van Bruggen (22) and Pratley (52).

Although the results obtained in the described experiments showed no correlation between active transport and cholinesterase activity, they do not preclude the possibility that cholinesterase is involved. Koblick's (33) modification of the Bonting and Featherstone (4) assay, which was used to determine the cholinesterase activity, was developed in 1956. Since then, more sensitive methods have been found (14). Before dismissing the hypothesis that cholinesterase is directly correlated with active sodium transport, it would be desirable to repeat the experiments using a more sensitive assay.

It would also be illuminating to repeat the experiments, measuring the cholinesterase activity from skins with the tela subcutanea removed. Although Koblick's experiments indicated that most of the cholinesterase activity found in the frog skin was located in the tela subcutanea (33), Franz and van Bruggen (22) showed that the frog skin still actively transported sodium after the tela subcutanea had been removed. The most recent experiments on the electrical profile of the frog skin indicate that the potential difference between the outside and the inside of the skin is formed by a series of jumps beginning just beneath the corium and continuing to the basement membrane, thus pointing to the epidermis as the location of the sodium pump and not the tela subcutanea. Koblick
(33) also showed that some cholinesterase activity still remained in the skin after the tela subcutanea had been removed. It is possible that only a very small amount of cholinesterase is needed for active transport and if that is assumed to be the case, it is possible that the amount of cholinesterase in the epidermis of the skin would vary with varying transport activity. Since the amount of cholinesterase in the epidermis is small compared to that in the tela subcutanea, this variation very likely would not be picked up by the method which was used.

Also, because cholinesterase has been implicated in sodium transport, one might expect to find acetylcholine in the tissue. Koblick (33) reported that the tissue controls never gave a readable color. However, a small amount of acetylcholine was consistently found in the tissue blanks which were run here.

In a few of the frog skins the sodium influx was measured using radioactive sodium-22 in order to verify that the current was an accurate measure of sodium transport. As indicated in the section giving the results, it was found that the net sodium flux measured with a microammeter on short-circuited skins was considerably lower than the sodium influx. Ussing and Zerahn (64) showed that for the short-circuited skin, the two values are nearly equivalent and later experiments by them and others have verified this
observation in all but a few instances. Bricker (6) noticed that when potassium Ringer's was substituted for sodium Ringer's as the solution bathing the inside of the frog skin, the net sodium flux as measured by radioisotopes was very much greater than that calculated from the short-circuit current. They attributed the difference to the efflux of potassium, for when the values for potassium efflux were subtracted from the net sodium flux, the net sodium flux was about equal to that calculated from the short-circuit current. Zadunaiskey and Candia (68) working on the frog Leptodactylus ocelatus also reported net transport values of sodium determined by radioisotopes that were much larger than the amount that could be accounted for by the current. After further experimentation, they concluded that this difference was due to chloride ions being actively transported in the same direction as the sodium ions. Another cause would be high sodium efflux, which could result from exchange diffusion.

Possibly one of these mechanisms would explain the discrepancies which were observed here. The last two are most likely and the active transport of chloride ions seems to be the more likely of the two. Experiments using radioisotopes and experiments in which the chloride ion was replaced by a slowly diffusing anion such as sulfate would have to be performed in order
to determine what was causing the lowered current. It might also
prove interesting to measure the chloride flux in animals which had
been treated in ways which would alter the rate of active transport.
The results obtained might give information concerning the relation-
ship between the sodium movement and the chloride movement and
also the effects of aldosterone on the movement of each ion.
SUMMARY

By salt depleting the animals or by injecting them with aldosterone, I was able to increase the active transport of sodium across the isolated skin. The potential difference was increased by 37% and 43% respectively and the net sodium flux by 69% and 62%. Exposure of frogs to 50mM NaCl did not produce a significant change in the rate of sodium transport. Although the resistance of the skins did not show a significant change as a result of the treatments, they did show trends. The skins from salt-depleted and aldosterone-injected frogs are less resistant than the controls. There was no correlation between the cholinesterase activity in the skin and the rate of active transport of sodium across the skin.
BIBLIOGRAPHY


