

AN ABSTRACT OF THE THESIS OF

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Title: NA-K-ACTIVATED ADENOSINETRIPHOSPHATASE AND  
SODIUM TRANSPORT IN LARVAL RANA CATESBEIANA

Abstract approved: Redacted for privacy  
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Sodium-potassium-activated adenosinetriphosphatase (Na-K-ATPase, E. C. 3.6.1.3) was measured in microsomal fractions of gills, kidney, skin, intestine, liver and skeletal muscle of larval and adult Rana catesbeiana. Emphasis was placed on gills and skin because of their possible role in transepithelial ion transport. Gill Na-K-ATPase requires both Na and K as well as Mg for maximum activity and is inhibited by  $10^{-4}$  M ouabain. The apparent  $K_m$  values with respect to ATP (substrate) and Na and K are 0.6, 4.5 and 2.0 mM respectively. The pH optimum is 7.5, the temperature optimum is  $45^{\circ}\text{C}$  and the energy of activation is 12,100 cal/mole ( $10^{\circ}$ - $20^{\circ}\text{C}$ ).

In the early stages of metamorphosis (stages X-XVIII) the mean gill Na-K-ATPase activity was  $2.41 \pm 0.12$   $\mu\text{moles}/\text{P}_i/\text{mg}$  pr/hr. Skin ATPase activity during this period is low ( $0.53 \pm 0.06$   $\mu\text{moles } \text{P}_i/\text{mg}$  pr/hr). After stage XVIII gill Na-K-ATPase declines and disappears at stage XXV whereas skin activity triples by this

stage. This is correlated with the appearance of the transepithelial pump in the skin.

The mean influx of Na, measured with  $^{22}\text{Na}$ , into tadpoles adapted to dechlorinated tap water (0.2 mM NaCl) is 1.50  $\mu\text{M}/10\text{g-hr}$ . This translocation of Na is thermodynamically active. Prior salt depletion of tadpoles by immersion in flowing distilled water for eight days activates the Na pump and is associated with a 36% increase in branchial Na-K-ATPase. Prior salt loading by immersion in 25 mM NaCl suppresses the influx from tap water and decreases Na-K-ATPase by 46%.

A single injection of aldosterone (2  $\mu\text{g}/\text{g}$ ) increases active influx of Na by 83% and elevates Na-K-ATPase by 19%. Single injection of cortisol affected neither the influx of Na or activity of branchial Na-K-ATPase, however multiple injections caused an 81% elevation in Na influx and a 21% increase in Na-K-ATPase. Single or multiple injections of prolactin did not affect the influx of Na or Na-K-ATPase. However multiple injections of prolactin decreased the efflux of Na. Single or multiple injection of cortisol plus prolactin increased Na influx (81%) and Na-K-ATPase activity (42%). Finally, thyroxin (single injection) had no effect on Na influx or Na-K-ATPase activity.

The data support the hypothesis that the gills of tadpoles are

an important site of active transepithelial transport of Na and provide further evidence that this transport is correlated with Na-K-ATPase. Certain hormones involved in ionoregulation may act by directly or indirectly affecting Na-K-ATPase activity in epithelia.

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Na-K-Activated Adenosinetriphosphatase  
and Sodium Transport in Larval Rana catesbeiana

by

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## LIST OF ABBREVIATIONS

|           |                           |
|-----------|---------------------------|
| ADP       | Adenosine 5'-diphosphate  |
| ATP       | Adenosine 5'-triphosphate |
| ATPase    | Adenosinetriphosphatase   |
| CTP       | Cytidine 5'-triphosphate  |
| g         | Gram                      |
| GTP       | Guanosine 5'-triphosphate |
| hr        | Hour                      |
| ITP       | Inosine 5'-triphosphate   |
| $K_m$     | Michaelis constant        |
| M         | Mole                      |
| mg        | Milligram                 |
| $M_i$     | Influx                    |
| $M_o$     | Efflux                    |
| $M_{net}$ | Net flux                  |
| Pi        | Inorganic phosphate       |
| pr        | Protein                   |
| $\mu$     | Micro                     |
| UTP       | Uridine 5'-triphosphate   |
| V         | Volume                    |
| W         | Weight                    |

Na-K-ACTIVATED ADENOSINETRIPHOSPHATASE  
AND SODIUM TRANSPORT IN LARVAL Rana catesbeiana

INTRODUCTION

A larval frog is typically faced with the same problem of osmotic and ionic regulations as other fresh water animals. Living in a hypoosmotic environment, animals lose sodium and chloride passively in urine and by diffusion along the electrochemical gradient across the skin. Most fresh water animals have an ability to compensate for this loss by transporting ions from dilute external media across the boundary membranes against an electrochemical gradient. In adult frogs this ability is well developed with both Na and Cl being actively transported across the skin (Jorgensen et al., 1954). However, the larval skin is incapable of active transport of Na and Cl (Taylor and Barker, 1965a; Alvarado and Moody, 1970). Since cells capable of active transport of ions exist in the gills of fish, it was suggested as early as 1939 by Krogh that external ionic transport might occur in amphibian gills as well. Surprisingly, over a period of 30 years no work was done to search for such a mechanism in the gills of larval amphibians. In 1970, Alvarado and Moody demonstrated that bullfrog tadpoles actively transport Na and Cl ions into their body fluid from dilute external media, and gills are the major site of this transport.

It is well established that the enzyme sodium-potassium-activated adenosinetriphosphatase (Na-K-ATPase; E. C. 3 6 1 3) is intimately involved in the transport of ions across cell membranes and epithelia (Skou, 1965; Katz and Epstein, 1968; Bonting, 1970). This enzyme was first discovered by Skou (1957) in the peripheral nerves of crabs. The biochemical characteristics and intracellular localization in various animal tissues of the enzyme are closely correlated with the activity of the sodium active transport system in such tissue (Bonting and Caravaggio, 1963; Bonting and Canady, 1964; Utida et al., 1971). It is important to note that the presence of this enzyme in an epithelium does not constitute proof that the tissue participates in transepithelial transport. The enzyme is also involved in the extrusion of Na from cells, an activity carried on by most cells including those which make up the epithelium. Therefore, to relate the presence of the enzyme to transepithelial transport, one must obtain additional information.

The intensity of active transepithelial transport is a physiological variable subject to elaborate homeostatic control. Endocrine mediation is involved. The hypothalamic-neurohypophysial complex and the adrenal cortex have both been implicated (Alvarado and Johnson, 1966; Alvarado and Kirschner, 1964). However, the mechanisms have not been elucidated at the organismic or cellular levels. In addition, prolactin, a hormone derived from the anterior

pituitary, has a pronounced effect on salt balance in fishes (Chan et al., 1968). Its role in amphibian hydromineral metabolism remains obscure (Crim, 1972).

The endocrine and neuroendocrine pathways involved in regulating release of the appropriate hormones are, doubtlessly, complex. However, in view of the apparent identity of the transport system with Na-K-ATPase it seems reasonable to start with the hypothesis that the hormones act directly, or indirectly on this enzyme system. Presumably they could promote synthesis of the enzyme or retard degradation. Alternatively they could "modulate" the activity of existing enzyme.

The aims of this study are (1) to provide evidence that the Na-K-ATPase in the gills of larval anurans is involved in transepithelial transport; (2) to characterize the enzyme and compare it with enzyme derived from other tissues; and (3) to compare the effects of salt-depletion, salt loading and injection of various hormones on Na transport and Na-K-ATPase in gills.



## MATERIALS AND METHODS

### Animals

Larval and adult R. catesbeiana were collected near Corvallis, Oregon. Tadpoles of various stages of development were classified as described by Taylor and Kollros (1946) and kept unfed at 12°C in dechlorinated tap water which contained about 0.1 mM NaCl. Animals were brought to room temperature (23°C) one week before use.

Salt-depleted animals were prepared by placing about eight animals in a container with distilled water flowing through at a rate of about 10 liters/day. Corresponding control animals were placed in flowing dechlorinated tap water for the same period of time. Animals were salt-loaded in flowing tap water containing 25 mM NaCl.

### Fluxes

Sodium influx of tadpoles was measured with  $^{22}\text{Na}$  as described by Alvarado and Moody (1970). The isotope (about 1 microcurie) was added to a 100 ml bath of dechlorinated tap water (NaCl = 0.1 mM). At specific intervals, 5.0 ml samples were withdrawn. One milliliter was transferred to a planchet and evaporated to dryness. The time required to accumulate 10,000 counts was determined with a gas-flow Geiger counter. Bath samples were also analyzed for Na concentration by flame photometry and the net flux ( $M_{\text{net}}$ ) was

determined from these values:

$$M_{\text{net}} = \frac{10V (C_t - C_o)}{t \times \text{wt}} \quad (1)$$

Where:  $M_{\text{net}}$  = net flux in  $\mu\text{Moles}/10\text{g-hr}$   
 $C_t$  = concentration of Na in  $\mu\text{Moles}/\text{ml}$  at  
time  $t$   
 $C_o$  = concentration of Na in  $\mu\text{Moles}/\text{ml}$  at  
time zero  
 $V$  = bath volume (ml)  
 $\text{wt}$  = weight of animals in grams.

The influx was calculated from equation (2) for animals not in a steady state and from equation (3) for animals in a steady state (Jorgensen et al., 1946).

$$M_i = M_{\text{net}} \left[ \frac{\log Y_o/Y_t}{\log A_o/A_t} \right] \quad (2)$$

$$M_i = \frac{CV}{t} \ln \frac{Y}{Y_o} \quad (3)$$

The efflux is given by:

$$M_o = M_{\text{net}} - M_i \quad (4)$$

### Tissues

Animals were anesthetized in 0.1% Finquel (Tricaine methane sulfonate) with the pH adjusted to 7.0 with sodium bicarbonate. After they were immobilized each animal was rinsed in distilled water. The gills, kidneys and liver were removed and blotted on tissue paper to remove adherent blood. Skin was removed from the ventral abdominal region and placed on a piece of Parafilm. Muscle and some of the connective tissue adhering to the corium side were removed by gentle scraping. The corium was not removed. The skin was then cut into small pieces. Muscle was taken from the tail region of larvae stages X to XVIII and from the hind leg of older tadpoles and adults. A section (6") of intestine was removed and flushed with 100 mM NaCl to wash off food and fecal materials.

### Preparation of Enzyme

All tissues and reagents were kept on ice. About one volume of tissue and three volumes of 0.1% deoxycholate (sodium salt or deoxycholic acid--depending on the desired medium) in 0.25 M sucrose, 1 mM EDTA (ethylenediamine tetraacetic acid) and 20 mM Tris-HCl buffer (pH 8.2) were homogenized for three minutes in a Potter-Elvehjem glass homogenizer driven by an electric drill (800 rpm). More of the homogenization medium was added to bring the dilution to about one volume of tissue to 10 volumes of solution and

this mixture was homogenized an additional minute. After the homogenate was passed through cheesecloth, it was centrifuged at 10,000 x g for 10 minutes at 2°C. The supernatant then was centrifuged at 80,000 x g for one hour at 2°C. The pellet was resuspended in 1 mM EDTA to bring the concentration of protein to approximately 1 - 2 mg/ml.

To obtain a mitochondrial fraction the homogenate was passed through cheesecloth and then centrifuged at 800 x g for 10 minutes. The supernatant was then recentrifuged at 10,000 x g. The mitochondrial sediment was resuspended in 1 mM EDTA, and the supernatant was treated as in the regular procedure for microsomal enzymatic preparation.

The assay for enzyme activity was performed within eight hours after killing the animals except for the study of the effect of storage on enzyme activity.

#### Enzymatic Assay Systems

The complete reaction mixture contained 1 mM EDTA, 5 mM  $MgCl_2$ , 20 mM KCl, 100 mM NaCl, 3 mM ATP (Na or Tris salts), 50 mM Tris-HCl buffer (pH 7.5) and 100 - 300  $\mu$ g of enzyme protein solution (0.2 ml). The tubes were set up in duplicate pairs; two tubes containing  $10^{-4}$  M ouabain, and the other two no ouabain. The final volume in each tube was 2 ml. Before adding ATP, the tubes

were incubated at the desired temperature (usually 25°C) for five minutes. The reaction was started by adding ATP. Blanks were run against the substrate and enzyme solution. Time-course studies revealed that the hydrolysis of ATP was linear for 30 minutes. For most studies the reactions were stopped after 20 minutes by adding 1 ml of 20% trichloroacetic acid to each tube. For the kinetic studies time courses were run for each substrate concentration. After standing for 10 minutes the assay system was centrifuged for 10 minutes (1,500 x g). The supernatant was assayed for liberated inorganic phosphate.

#### Inorganic Phosphate Determination

Inorganic phosphate was determined according to the method of Stanton (1968). One ml of supernatant was aspirated into an auto-dilutor and was dispensed with 7.6 ml of water into appropriate tubes containing 1.0 ml of 2.5% ammonium molybdate in 5 N sulfuric acid. Then, 0.4 ml of 0.25% 1,2,4-2aminonaphtho sulfonic acid in sulfite-metabisulfite buffer was added and mixed well. The optical density was read at exactly 20 minutes at 815 m $\mu$ . Potassium dihydrogen phosphate was used as a standard. Protein concentration was determined as described by Lowry et al., 1951.

## RESULTS

### Enzyme Purification

The combination of differential centrifugation, treatment with deoxycholate and NaI (Matsui and Schwartz, 1966) were employed for the enzyme preparation (Figure 1). Data for the purification of Na-K-ATPase from gills of larval R. catesbeiana are summarized in Table 1. A very low specific activity of ouabain-sensitive ATPase and high ratio of Mg-ATPase to Na-K-ATPase were observed in the crude homogenate with 0.1% deoxycholate. The enzyme specific activity was increased progressively in each step of centrifugal separation. The Na-K-ATPase is 2 and 10 fold higher in the supernatant after 10,000 x g and in sediment after 80,000 x g respectively. Treatment of enzyme with NaI resulted in further elevation of Na-K-ATPase and suppression of Mg-ATPase activities. Similar results were found in enzyme prepared from erythrocytes (Nakao et al., 1963) and brain (Nakao et al., 1965). Pretreatment of homogenate with 1.5 M urea for 30-60 minutes also suppresses Mg-ATPase activity (Bakkeren and Bonting, 1968). The percent recovery of protein after 80,000 x g is  $4.5 \pm 0.4$  and  $0.9 \pm 0.1\%$  after NaI treatment. It has been reported that the percent recovery of protein in microsomal fractions after 65,000 x g for cardiac muscle is 3.3% (Matsui and Schwartz, 1966), and in turtle bladder is 6.0% (Shamoo and Brodsky,

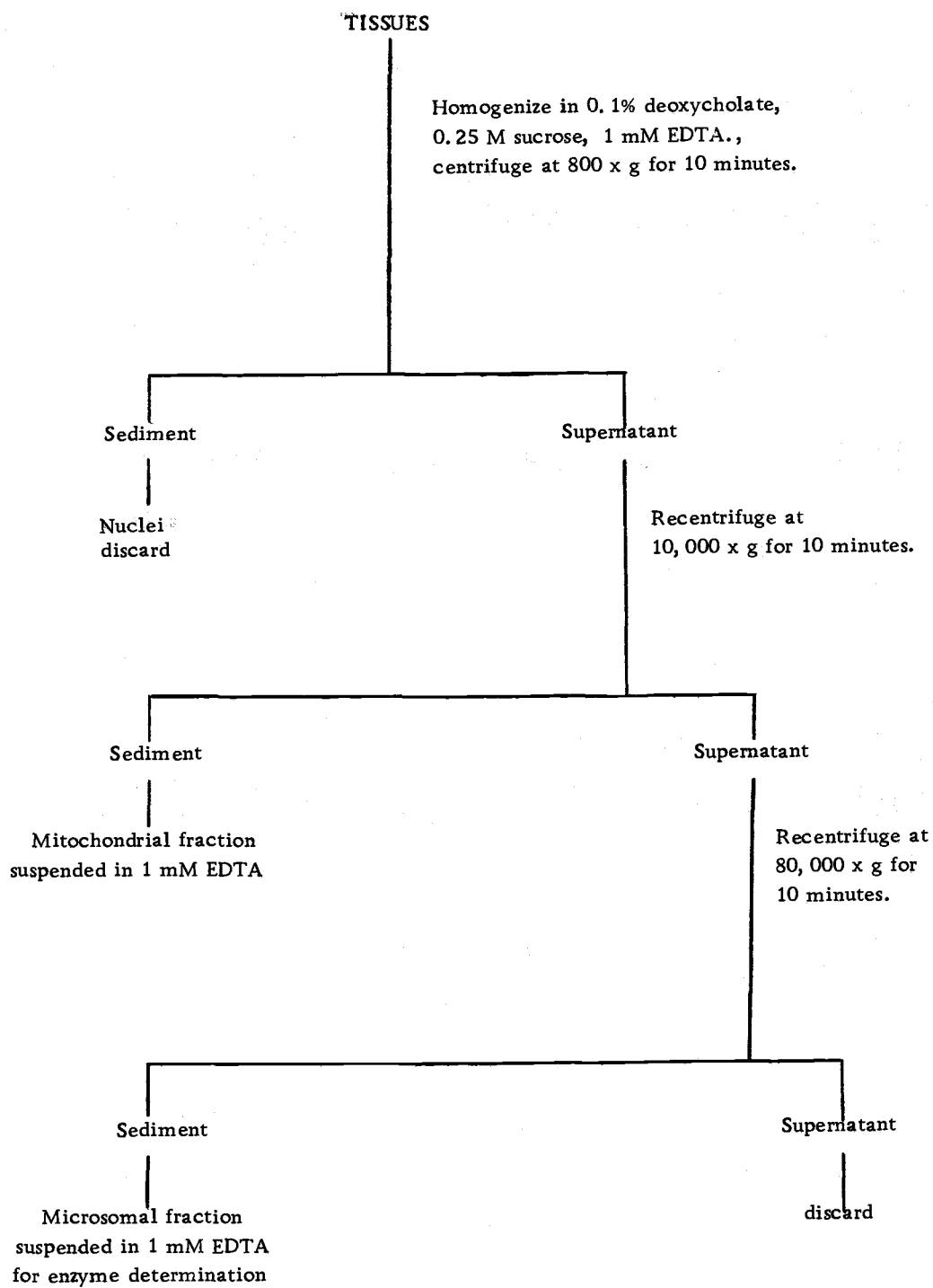


Figure 1. Flow sheet diagram of the purification of Na-K-ATPase.

Table 1. Purification of Na-K-ATPase from gills of larval R. catesbeiana.

| Treatment                       | Na-K-ATPase      | Mg-ATPase        | Mg-ATPase/<br>Na-K-ATPase | Protein          |                 | % Recovery of activity |               |
|---------------------------------|------------------|------------------|---------------------------|------------------|-----------------|------------------------|---------------|
|                                 |                  |                  |                           | mg               | % Recovery      | Na-K-ATPase            | Mg-ATPase     |
| Homogenate with<br>deoxycholate | 0.25             | 3.99             | 16.0                      | 225.1            | 100             | 100                    | 100           |
|                                 | $\frac{+}{0.03}$ | $\frac{+}{0.12}$ | $\frac{+}{1.7}$           | $\frac{+}{3.7}$  |                 |                        |               |
| Supernate after<br>10,000 x g   | 0.49             | 6.01             | 12.3                      | 110.8            | 49.2            | 87                     | 74            |
|                                 | $\frac{+}{0.06}$ | $\frac{+}{0.75}$ | $\frac{+}{0.7}$           | $\frac{+}{11.5}$ | $\frac{+}{5.7}$ | $\frac{+}{9}$          | $\frac{+}{5}$ |
| Sediment after<br>80,000 x g    | 2.60             | 26.63            | 10.2                      | 10.1             | 4.5             | 47                     | 30            |
|                                 | $\frac{+}{0.14}$ | $\frac{+}{0.56}$ | $\frac{+}{0.5}$           | $\frac{+}{0.7}$  | $\frac{+}{0.4}$ | $\frac{+}{6}$          | $\frac{+}{3}$ |
| NaI treatment                   | 7.42             | 15.63            | 2.1                       | 2.2              | 0.9             | 29                     | 4             |
|                                 | $\frac{+}{1.15}$ | $\frac{+}{1.08}$ | $\frac{+}{0.4}$           | $\frac{+}{0.2}$  | $\frac{+}{0.1}$ | $\frac{+}{5}$          | $\frac{+}{1}$ |



1970). Since the recovery of protein after NaI treatment in gills of tadpoles is only  $0.9 \pm 0.1\%$ , it is very difficult to apply this step of enzyme purification in routine preparations which start out with only 100 to 200 mg of tissue. Recently, Kline et al. (1971) have demonstrated that the kinetic parameters of Na-K-ATPase are not influenced by the state of purity or differences in purification techniques. Since this study is primarily concerned with the physiological role of the enzyme in ion transport and less with detailed biochemical characterization, the microsomal sediment after 80,000 x g was used throughout this study.

Some other detergents have been used as substitutes for deoxycholate. The activities of Na-K-ATPase and Mg-ATPase in gills and kidney of tadpoles after treatment with these detergents are shown in Table 2. At 0.1% (w/v) detergents in 0.25 M sucrose and 1 mM EDTA, deoxycholate gives highest activity of both Na-K-ATPase and Mg-ATPase. The activity of Na-K-ATPase is decreased in the following order: deoxycholate > Brij-35 > Tergitol > Triton x-100. Different concentrations of deoxycholate have different effects on enzyme activities (Jorgensen et al., 1971) so that the order of enzyme activity might be different if the concentration of Brij-35, Tergitol, and Triton x-100 are varied. Using the detergent Lubrol and treatment with NaI, followed by salt and isoelectric precipitation, ultracentrifugation and carboxymethyl cellulose chromatography,

Table 2. ATPase activities in the microsomal fraction of gills from tadpoles after treatment with various detergents. The concentration of detergents is 0.1% (W/V).

| Detergent    | ATPase activity,<br>$\mu\text{moles P}_i/\text{mg pr/hr}$ |               |               |               |
|--------------|---|---------------|---------------|---------------|
|              | Gills   |               | Kidney        |               |
|              | Na-K  | Mg            | Na-K          | Mg            |
| Deoxycholate | 2.28  | 23.70         | 12.53         | 20.95         |
|              | $\pm$<br>0.15   | $\pm$<br>1.27 | $\pm$<br>1.04 | $\pm$<br>1.02 |
| Triton X-100 | 0.19  | 6.18          | 0.28          | 6.56          |
|              | $\pm$<br>0.06   | $\pm$<br>0.42 | $\pm$<br>0.02 | $\pm$<br>0.40 |
| Tergitol     | 0.28  | 8.42          | 0.68          | 7.78          |
|              | $\pm$<br>0.05   | $\pm$<br>0.44 | $\pm$<br>0.12 | $\pm$<br>0.78 |
| Brij-35      | 1.54  | 17.72         | 7.48          | 14.24         |
|              | $\pm$<br>0.08   | $\pm$<br>0.94 | $\pm$<br>0.51 | $\pm$<br>1.84 |

Kahlenberg et al. (1969) have achieved a high degree of purification of brain Na-K-ATPase. Recently, specific activity of 1500  $\mu\text{mole}/\text{mg pr/hr}$  was obtained by rate-zonal centrifugation from the outer medulla of rabbit kidney (Jorgensen et al., 1971).

### General Characteristics of Tadpole Gills ATPases

#### Location of the ATPases in the Gill Epithelium

Table 3 shows the activities of Na-K-ATPase and Mg-ATPase in the tadpole gill epithelium in crude homogenate, mitochondrail and microsomal fractions. Na-K-ATPase activity is highest in the microsomal fraction; crude homogenate and mitochondrail activities represent 13 and 73% of microsomal activity respectively. The microsomal sediment from rat brain after differential centrifugation was shown under the electron microscope to be the broken cell membranes (Hanzon and Toshi, 1959).

#### Quabain Sensitivity

Since the observation of Schatzmann (1953), Koefoed-Johnson (1958), and Whittam and Wheeler (1961), that cardiac glycosides inhibit cation transport, it has been well documented that these steroids are specific inhibitors of the Na-K-ATPase which lends strong support to the assumption that the enzyme system is involved in the active transport of Na and K. Of these cardiac glycosides, ouabain is most widely used because of its solubility in water.

Table 3. Distribution of ATPase activities in gills of R. catesbeiana.

| Fraction                     | ATPase activity, $\mu\text{moles P}_i/\text{mg pr/hr}$ |                  |
|------------------------------|--|------------------|
|                              | Na-K   | Mg               |
| Homogenate with deoxycholate | $0.33 \pm 0.02$  | $3.82 \pm 0.11$  |
| Mitochondrial                | $1.81 \pm 0.07$  | $13.32 \pm 0.34$ |
| Microsomal                   | $2.48 \pm 0.15$  | $24.23 \pm 1.30$ |

Figure 2 shows the inhibition curve for ATPase from tadpole gills. The inhibitory effect of ouabain is seen at very low concentrations ( $10^{-10}$  M) with complete inhibition at  $10^{-4}$  M. Fifty percent inhibition occurs at about  $2.4 \times 10^{-7}$  M ouabain.

#### Requirement of Na and K

Na-K-ATPase differs from other ATP-hydrolyzing enzymes in that in addition to Mg it requires both Na and K for maximum activity (Skou, 1957). In enzyme prepared from the gills of R. catesbeiana, removal of Na and K or Na or K reduces the total ATPase activity to the same extent as adding  $10^{-4}$  M ouabain (Table 4). Ammonium ion can substitute for K, but not Na. Substitution of K with  $\text{NH}_4\text{Cl}$  and  $(\text{NH}_4)_2\text{SO}_4$  results in 76% and 78% of original Na-K-ATPase activity.

Table 4. Ionic activation of ATPase activity.

| Assay system  | ATPase,<br>$\mu$ M/mg pr/hr |            | Ouabain<br>sensitive<br>activity | %   |
|---|-----------------------------|------------|----------------------------------|-----|
|   | - Ouabain                   | + Ouabain* |                                  |     |
| MgCl <sub>2</sub> + NaCl + KCl  | 28.38                       | 25.93      | 2.45                             | 100 |
| MgCl <sub>2</sub> + NaCl  | 25.93                       | 25.93      | 0                                | 0   |
| MgCl <sub>2</sub> + KCl   | 25.93                       | 25.93      | 0                                | 0   |
| MgCl <sub>2</sub>   | 25.93                       | 25.93      | 0                                | 0   |
| MgCl <sub>2</sub> + NaCl + NH <sub>4</sub> Cl   | 27.79                       | 25.93      | 1.86                             | 76  |
| MgCl <sub>2</sub> + KCl + NH <sub>4</sub> Cl  | 25.93                       | 25.93      | 0                                | 0   |
| MgSO <sub>4</sub> + Na <sub>2</sub> SO <sub>4</sub> + K <sub>2</sub> SO <sub>4</sub>                  | 27.37                       | 24.85      | 2.42                             | 99  |
| MgSO <sub>4</sub> + Na <sub>2</sub> SO <sub>4</sub> + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 26.76                       | 24.85      | 1.91                             | 78  |
| MgSO <sub>4</sub> + K <sub>2</sub> SO <sub>4</sub> + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>  | 24.85                       | 24.85      | 0                                | 0   |
| MgSO <sub>4</sub>   | 24.85                       | 24.85      | 0                                | 0   |

\*10<sup>-4</sup> M

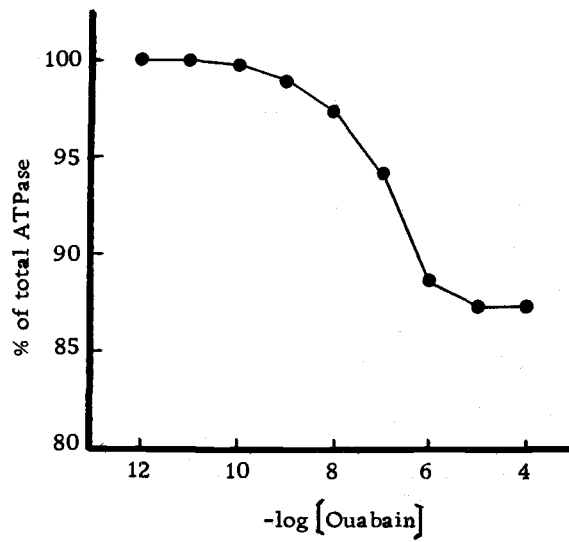


Figure 2. Effect of ouabain concentration (molar) on total ATPase in microsomal fraction of gill tissue from tadpoles of R. catesbeiana.

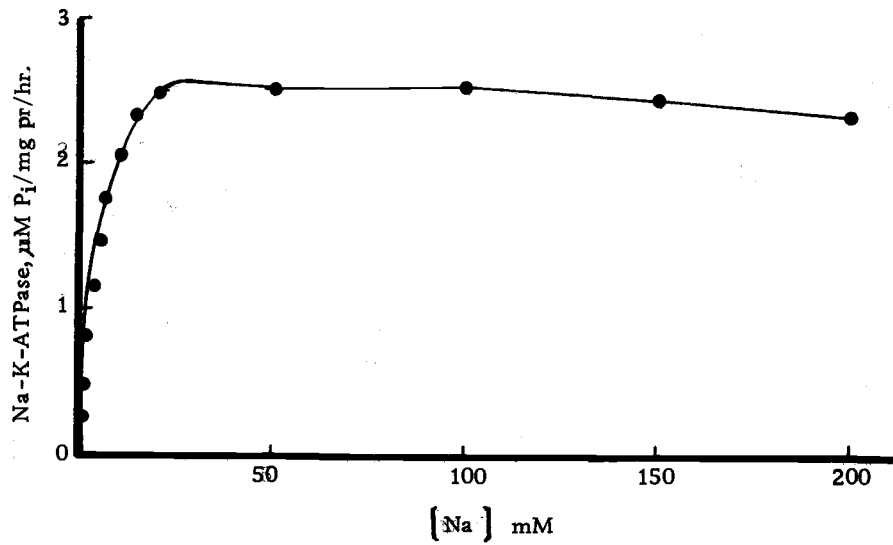


Figure 3. Na activation curve of Na-K-ATPase in microsomal fraction of gill tissue from tadpoles of R. catesbeiana.

A Na activation curve for Na-K-ATPase is shown in Figure 3. Sodium concentrations above 25 mM give maximum activation. Concentrations greater than 125 mM depress the Na-K-ATPase activity slightly. The half-maximum activation concentration,  $K_m$ , is 4.5 mM.  $K_m$  of  $[Na]$  for Na-K-ATPase seems to vary with the type of animal and tissue from 1.0 mM in guinea pig intestine (Taylor, 1962) to 12.5 in salt gland of herring gull (Bonting *et al.*, 1964).

Effect of K concentration on Na-K-ATPase activity is shown in Figure 4. The half maximum activation,  $K_m$ , is 2.0 mM compared to a value of 1.8 in nerve from crabs (Skou, 1957).

#### Substrate Specificity

The specificity of substrate of Na-K-ATPase is shown in Table 5. Only ITP gives a considerable degree of stimulation (29% of ATP), while CTP and GTP give only 18% and 9% stimulation, respectively. UTP was not tested. Na and K did not activate a system which had ADP as substrate. The  $K_m$  for ATP, calculated from Figure 5 for the Na-K-ATPase, is 0.6 mM while  $K_m$  for Mg-ATPase is 0.4 mM.

#### pH Dependency

Figure 6 shows a plot of Na-K-ATPase and Mg-ATPase activities versus pH of the assay system. Na-K-dependent activity shows a sharp peak at pH of 7.5. The Mg-dependent activity, on the other

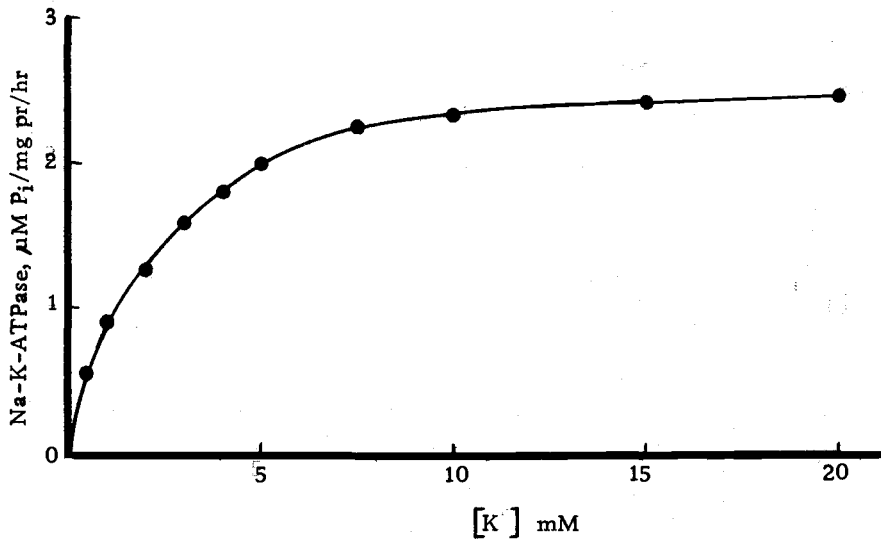


Figure 4. K activation curve of Na-K-ATPase in microsomal fraction of gill tissue from tadpoles of *R. catesbeiana*.

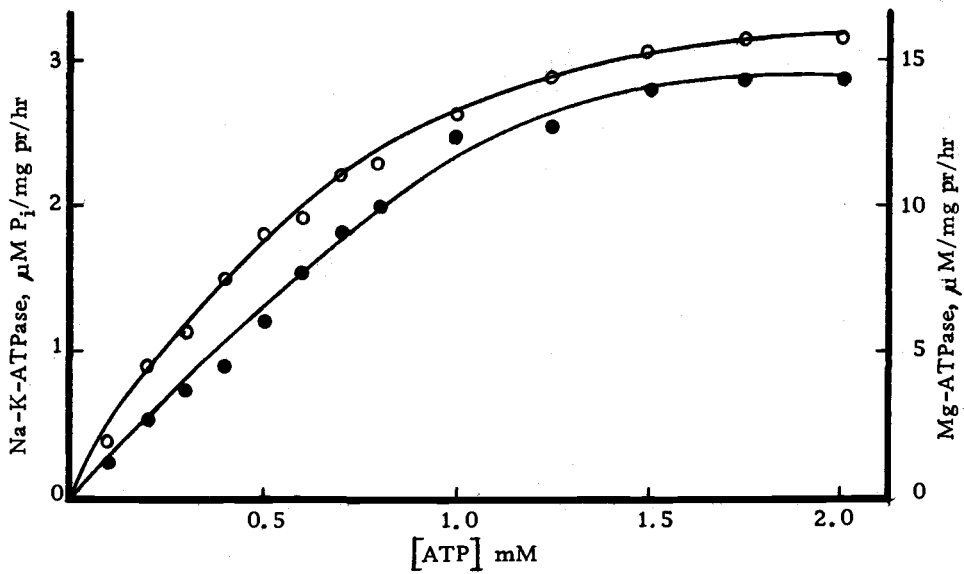


Figure 5. Effect of ATP concentration on Na-K-ATPase and Mg-ATPase in the microsomal fraction of gills of bullfrog tadpole. Solid circles are Na-K-ATPase, open circles Mg-ATPase.



Table 5. Substrate specificity of tadpole gill ATPase.

| Sub-<br>strate | Specific activity, $\mu\text{M}/\text{mg pr/hr}$ |                          |                                       | %<br>Activity<br>of Na-K-<br>ATPase |
|----------------|--|--------------------------|---------------------------------------|-------------------------------------|
|                | - Ouabain<br>(Total ATPase)                      | + Ouabain<br>(Mg-ATPase) | Ouabain<br>sensitive<br>(Na-K-ATPase) |                                     |
| ATP            | 27.05  | 24.62                    | 2.43                                  | 100                                 |
| ITP            | 17.14  | 16.43                    | 0.71                                  | 29                                  |
| CTP            | 13.21  | 12.69                    | 0.43                                  | 18                                  |
| GTP            | 20.00  | 19.78                    | 0.22                                  | 9                                   |
| ADP            | 5.24   | 5.24                     | 0                                     | 0                                   |

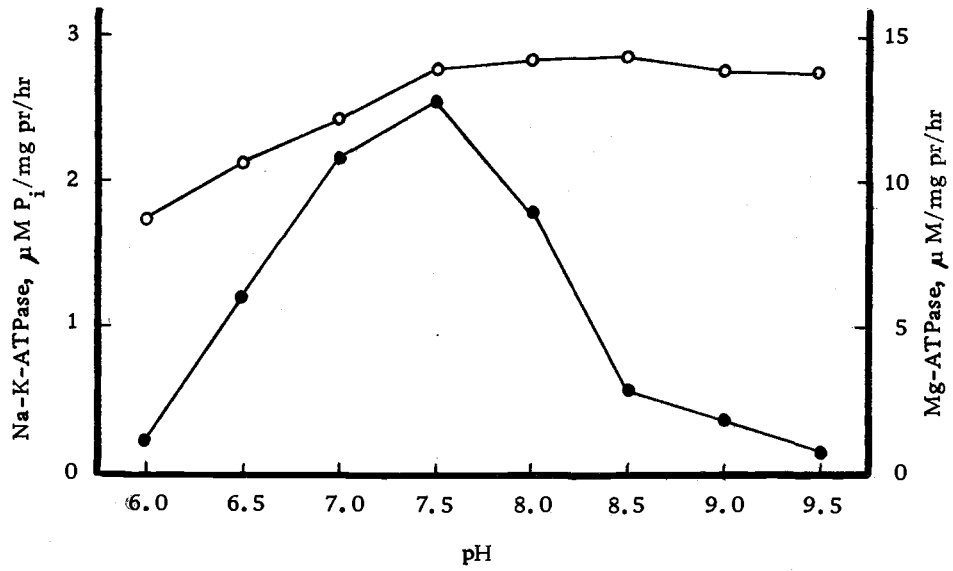


Figure 6. Effect of pH on Na-K-ATPase and Mg-ATPase in the microsomal fraction of gill tissue from tadpoles of *R. catesbeiana*. Solid circles are Na-K-ATPase, open circle Mg-ATPase.

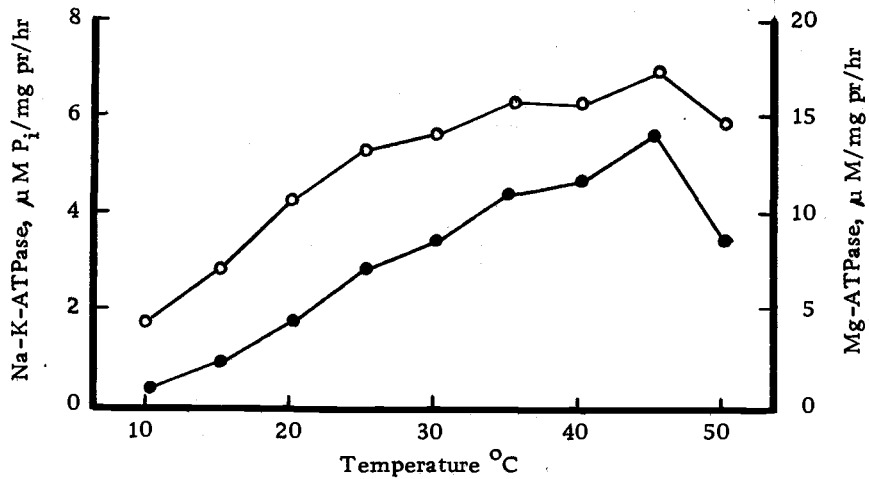


Figure 7. Effect of temperature on Na-K-ATPase and Mg-ATPase in the microsomal fraction of gill tissue from tadpoles of *R. catesbeiana*. Solid circles are Na-K-ATPase, open circle Mg-ATPase.

hand, shows a broad plateau between pH 6.0 and 9.0. Similar effects have been observed in other tissues (Shamoo and Brodsky, 1970; Matsui and Schwartz, 1966).

### Temperature Effect

The effects of temperature on the Na-K-ATPase and Mg-ATPase activities are shown in Figure 7. Both components of ATPase gave a similar response to temperature up to 45°C with the optimum activity at this point. Na-K-ATPase was much more sensitive to heat at 50°C than Mg-ATPase. The  $Q_{10}$  values between 25 to 40°C are 2.1 ( $E_A = 12,100$  cal/mole) for Na-K-ATPase and 1.6 for Mg-ATPase. These values are similar in magnitude to those reported for other tissues (Bonting and Carravagio, 1963; Shamoo and Brodsky, 1970).

### Stability of Enzyme

Figure 8 shows the effect of storage at -20°C and 4°C on ATPase activity. Storage for 48 hours at -20°C did not affect Na-K- or Mg activated ATPase. Forty-eight hours storage at 4°C resulted in a slight decrease in Na-K-ATPase and a sharp decrease in Mg-ATPase. Storage beyond 48 hours at -20°C increased activity in Na-K-ATPase and decreased in Mg-ATPase.

### Distribution and Changes in Na-K-ATPase Activity During Metamorphosis

Metamorphosis is a post embryonic developmental change of an animal "preparatory" to a change in environment. The biochemical

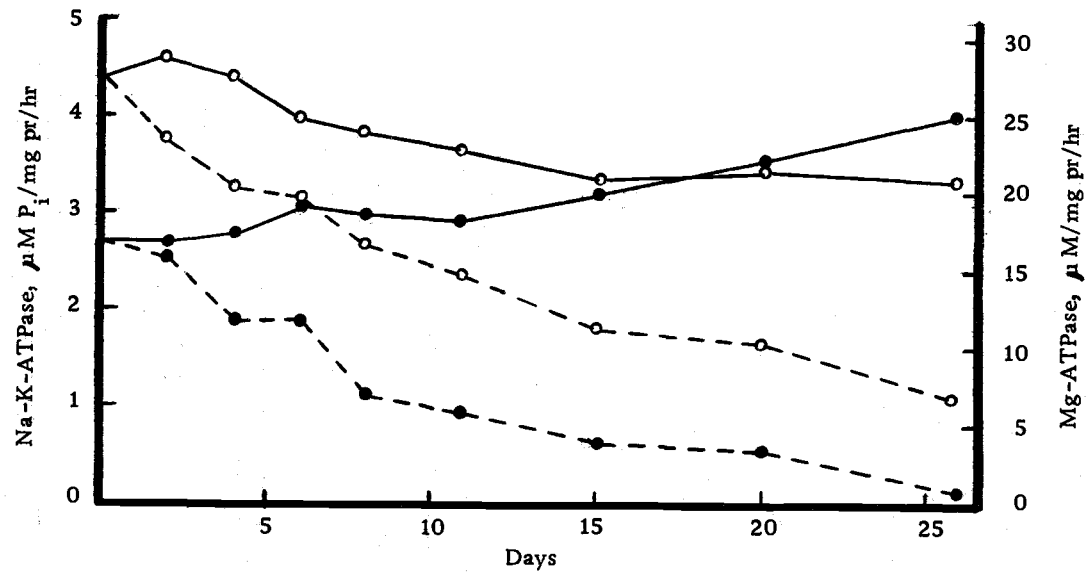


Figure 8. Storage effect on the ATPase in microsomal fraction of gill tissue from R. catesbeiana.  
 Na-K-ATPase at 4°C (●---●). Mg-ATPase at 4°C (○---○).  
 Na-K-ATPase at -20°C (●—●). Mg-ATPase at -20°C (○—○).

changes that occur during anuran metamorphosis usually appear to have direct or indirect adaptive value for the transition from entirely aquatic to semi-aquatic environments. Among the most important adaptive changes are the shift from ammonotelism to ureotelism, reflecting water availability; increasing serum albumin and other proteins, reflecting homeostasis and maintenance of circulatory volume; changes in biosynthesis and properties of hemoglobin for greater availability of oxygen; change in digestive enzymes for necessary adjustment to the alternation of diet; and tail regression and limb development leading to more powerful locomotion on land (Bennett and Frieden, 1962; Frieden and Just, 1970).

There have been a limited number of reports concerning either the presence or the changing of Na-K-ATPase activity during metamorphosis of anuran larvae. Such studies should yield further information relating to adaptive changes in enzymes at metamorphosis as well as provide further evidence for the involvement of Na-K-ATPase in active transport of Na.

#### Gill and Skin

Gills of tadpoles generally start to atrophy at stage XX and completely disappear at stage XXV. The gill Na-K-ATPase activity is at about the same level from stage X to stage XX, however, after stage XXI the activity is sharply decreased (Figure 9).

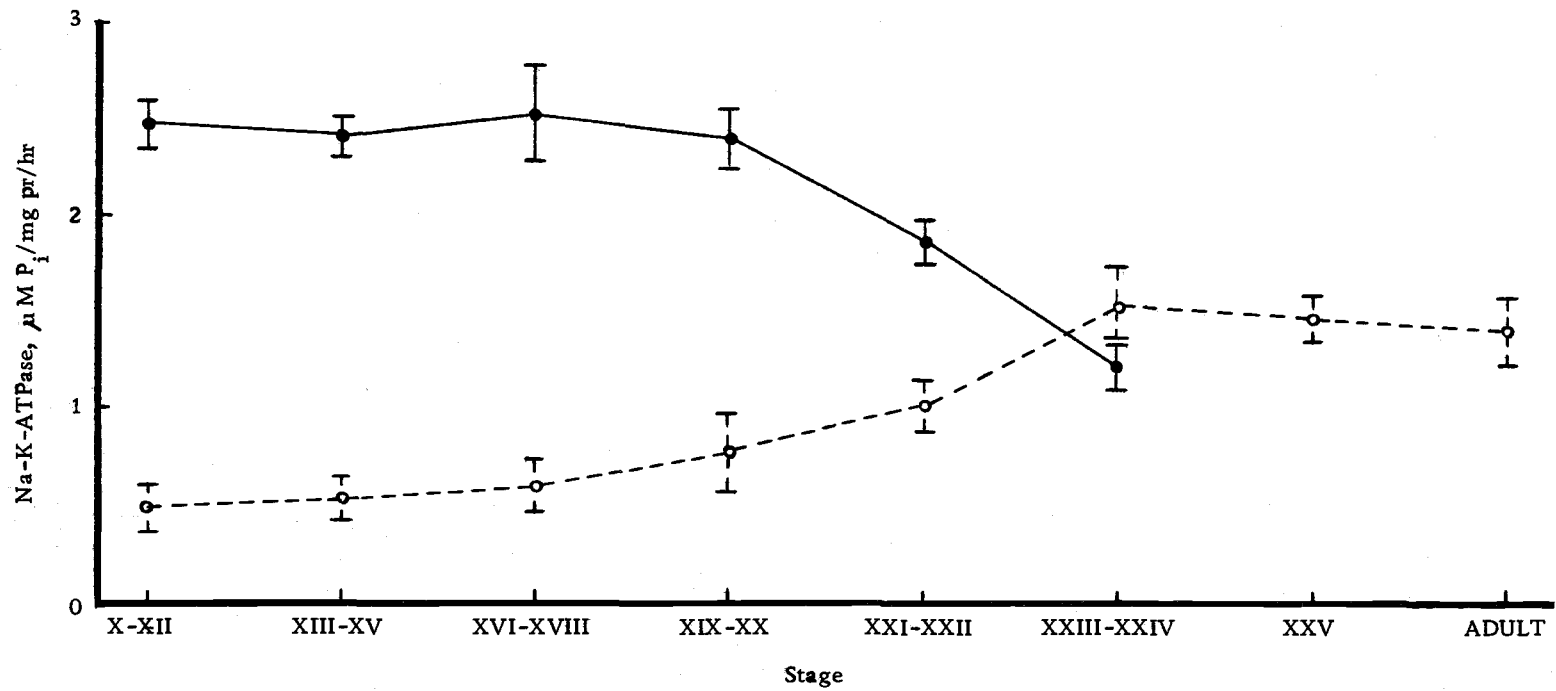


Figure 9. Na-K-ATPase activity during metamorphosis in microsomal fraction of gills (solid line) and skin (broken line) of *R. catesbeiana*.

Na-K-ATPase in the skin of adult R. catesbeiana is about three times higher than in larval skin. The skin of adult frog is known to actively transport Na but larval skin does not (Alvarado and Moody, 1970). The increase in skin Na-K-ATPase is clearly evident by stage XXIII. The active transport of Na in the skin of tadpoles appears at stage XXI (Taylor and Barker, 1965). The transepithelial potential across the skin was also found to appear at stage XXI (Alvarado and Moody, 1970). Therefore, the appearance of active transport in larval skin coincides with the increase Na-K-ATPase activity. It is shown from this study that there is an inverse correlation of Na-K-ATPase activities in the gills and skin of tadpoles. It is, then, suggested that during metamorphosis of anuran larvae there is a shift of the route of active transport of Na from gills to skin.

### Kidney

The occurrence of the Na-K-ATPase in the kidney has been reported in vertebrates from fish (Epstein et al., 1969) to mammals (Bonting et al., 1961). Tadpole kidney Na-K-ATPase activity is the highest among five tissues analyzed (Table 6), and the enzyme activity also increases as metamorphosis proceeds. This suggests an important role of the kidney Na-K-ATPase of tadpoles in Na reabsorption as in higher vertebrate (Katz and Epstein, 1968). In amphibians, most investigators have used the urinary bladder for study of ATPase and Na transport. There are no reports concerning

Table 6. Na-K-ATPase activity ( $\mu\text{M P}_i/\text{mg pr/hr}$ ) in microsomal fraction of kidney, intestine, muscle, and liver during metamorphosis of tadpoles R. catesbeiana.

| Tissue    | X-XII         | XIII-XV       | XVI-XVIII     | XIX-XX        | XXI-XXII      | XXIII-XXIV    | XXV           | ADULT         |
|-----------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Kidney    | 11.44         | 12.68         | 13.89         | 15.11         | 15.44         | 17.23         | 19.23         | 23.16         |
|           | $\pm$<br>0.68 | $\pm$<br>0.82 | $\pm$<br>0.36 | $\pm$<br>1.45 | $\pm$<br>1.50 | $\pm$<br>0.24 | $\pm$<br>1.24 | $\pm$<br>1.46 |
|           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           |
| Intestine | 1.66          | 1.63          | 1.32          | 1.18          | 1.58          | 1.32          | 3.44          | 9.54          |
|           | $\pm$<br>0.18 | $\pm$<br>0.29 | $\pm$<br>0.16 | $\pm$<br>0.19 | $\pm$<br>0.13 | $\pm$<br>0.49 | $\pm$<br>0.89 | $\pm$<br>0.61 |
|           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           |
| Muscle    | 0.55          | 0.57          | 0.60          | 1.07          | 1.00          | 0.79          | 1.01          | 1.40          |
|           | $\pm$<br>0.14 | $\pm$<br>0.12 | $\pm$<br>0.12 | $\pm$<br>0.27 | $\pm$<br>0.25 | $\pm$<br>0.16 | $\pm$<br>0.19 | $\pm$<br>0.31 |
|           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (8)           |
| Liver     | 0.09          | 0.16          | 0.25          | 0.67          | 0.60          | 1.05          | 0.75          | 1.82          |
|           | $\pm$<br>0.04 | $\pm$<br>0.08 | $\pm$<br>0.14 | $\pm$<br>0.14 | $\pm$<br>0.37 | $\pm$<br>0.18 | $\pm$<br>0.17 | $\pm$<br>0.53 |
|           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (4)           | (8)           |



the ATPase activities in each segment of amphibian nephron. However, Whittembury (1960) has demonstrated the presence of an active sodium pump in the proximal tubule of Necturus maculosus.

### Intestine

Tadpole intestine shows Na-K-ATPase activity at about the same level from stage X to XXIV. There is a significant increase from stage XXIV to adult ( $1.32 \pm 0.49$  to  $9.54 \pm 0.61$   $\mu\text{moles } P_i/\text{mg pr/hr}$ ). The change of enzyme activity coincides with the morphological shortening of intestine at stage XXIV. Young tadpoles drink at a rate of 0.1 ml/10g/hr and absorb about half of the fluid ingested (Alvarado and Moody, 1970). Since the Na concentration in normal pond water is about 0.1 to 0.2 mM, and the total influx of sodium in whole animals is about 1-2  $\mu\text{equiv}/10\text{g/hr}$ , tadpole intestines must play only a minor role in sodium absorption. The Na-K-ATPase in intestinal mucosal cells has been demonstrated in guinea pig (Taylor, 1962), rat (Taylor, 1963), goldfish (Smith, 1967), and eel (Oide, 1967).

### Muscle

Table 6 also shows Na-K-ATPase activity of the tail muscle of larvae from stage X to XVIII and hind leg muscle from stage XIX to adult. The activity of the hind leg is about double the activity in the tail muscle. Skeletal muscle also has a high K/Na ratio and large

membrane potential with the inside negative. Witt and Schatzmann (1954) demonstrated that cardiac glycosides cause inhibition of active Na efflux and loss of the resting potential. The Na-K-ATPase in the muscle is involved in the extrusion of Na from cells. Na-K-ATPase was demonstrated earlier in adult frog muscle (Bonting et al., 1962), frog toe muscle (Bonting and Caravaggio, 1963), and frog sartorius muscle (Corrie and Bonting, 1966).

### Liver

Na-K-ATPase activity in the liver during metamorphosis is shown in Table 6. There is a progressive increase in enzyme activity from larval stage X to adult. The activity at stages X - XII is very low ( $0.09 \pm 0.04$   $\mu\text{moles/mg pr/hr}$ ) as compared to activity at stages XXI - XXII ( $0.60 \pm 0.37$   $\mu\text{moles/mg pr/hr}$ ). There are numerous liver enzymes that increase in activity during metamorphosis (Weber, 1967; Frieden and Just, 1970). Only the urea-ornithine cycle enzymes have been studied in detail.

### Effects of External Salt Concentrations on Na-K-ATPase Activity

External salt concentration has a definite effect on ionic regulation in animals (Krogh, 1939). Salt depletion stimulates the active accumulation of Na and Cl in larval Ambystoma gracile (Alvarado and Dietz, 1970) and tadpoles, R. catesbeiana (Alvarado and Moody,

1970). On the other hand, when animals are exposed to a relatively higher concentration than natural pond water the active transport of ions is depressed (Alvarado and Moody, 1970; Kirschner et al., 1971).

#### Effect of Different External Na Concentrations

Figure 10 shows the effect of external Na concentration on tadpole gill Na-K-ATPase. The animals were adapted to each solution 14 days. The specific activity of Na-K-ATPase is inversely correlated to the external sodium concentration. It is indicated that tadpoles are able to "adjust" the enzyme activity according to their physiological needs. The rate of ionic loss is higher for animals living in "low salt" environments than in the higher salt environment. To compensate for the ionic loss, the function of the active ionic "pump" must be higher in animals that live in very dilute media.

#### Salt Load and Salt Depletion as a Function of Time

Figure 11 shows the Na-K-ATPase and Mg-ATPase activities in the gills of tadpoles which have been in distilled water or in 25 mM NaCl tap water for varying periods. Control animals were in tap water. Salt-depleted animals do not show any change of Na-K-ATPase activity on the fourth day of depletion. However by the eighth day of depletion, the enzyme activity increases from  $2.4 \pm 0.13$  to  $3.38 \pm 0.17$   $\mu\text{mole P}_i/\text{mg pr/hr}$  which is a significant difference ( $P < 0.01$ ). At the 12th and 16th day, the enzyme activity is slightly dropped, but

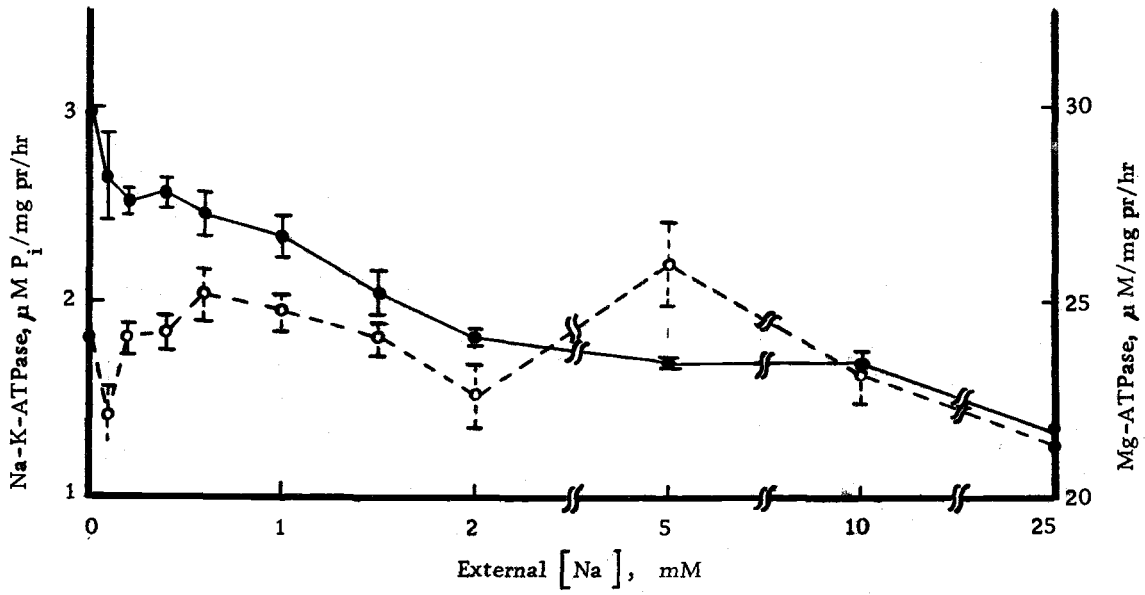


Figure 10. Effect of bath Na on gill ATPase. Na-K-(●—●) and Mg-(○---○)

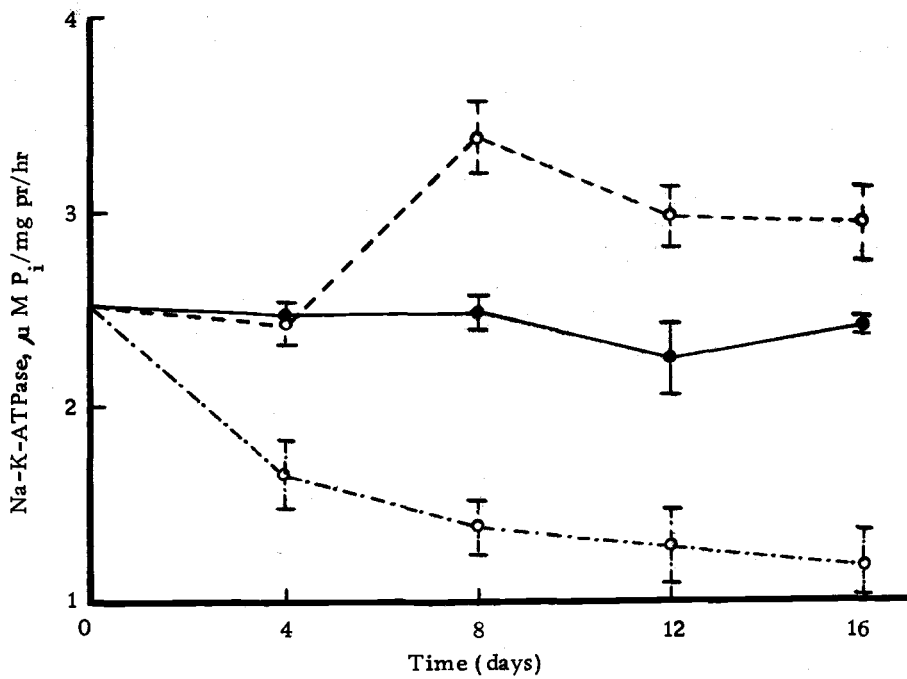


Figure 11. Tadpole gill Na-K-ATPase activity of salt loaded (○---○), salt depleted (○---○), and control (●—●) animals.

still is significantly higher than the control animals. Salt-loaded animals, on the other hand, show significant decreases of Na-K-ATPase from the fourth day and the enzyme activity decreases further as a function of time.

#### Effects of Salt Load and Salt Depletion on Kidney, and Skin Na-K-ATPase Activities

Table 7 shows the Na-K-ATPase and Mg-ATPase activities in kidney, and skin of control, salt loaded, and salt depleted animals after eight days of acclimation. There is a significant increase in kidney Na-K-ATPase in salt-depleted animals; and only a slight, insignificant, decrease in mean enzyme activity for salt-loaded animals. The stimulation of kidney Na-K-ATPase in salt-depleted animals may be related to increased Na retention in kidneys as suggested by Epstein et al. (1969).

#### Effects of Hormones on Sodium Transport and Adenosinetriphosphatase

##### Aldosterone

Aldosterone is the principal steroid produced by the interrenal gland of anurans (Carstensen et al., 1959, 1961; Ulick and Feinholtz, 1968). Crabbé (1963) has shown that salt balance in anurans is at least partially regulated by aldosterone. Injection of aldosterone increased active sodium transport in toad urinary bladder and ventral skin of Bufo marinus both in vitro and in vivo (Crabbé, 1961a, 1961b;

Table 7. ATPase activities in kidney and skin after animals have been salt depleted or salt loaded for eight days.

| Tissue | Enzyme      | Enzyme activity, $\mu\text{moles P}_i/\text{mg pr/hr}$ |                     |                     |
|--------|-------------|--|---------------------|---------------------|
|        |             | Control  | Salt depleted       | Salt loaded         |
| Kidney | Na-K-ATPase | $12.65 \pm 0.87(4)$                                    | $15.65 \pm 1.27(8)$ | $11.18 \pm 0.68(8)$ |
|        | Mg-ATPase   | $28.86 \pm 1.00(4)$                                    | $33.58 \pm 0.95(8)$ | $32.09 \pm 2.40(8)$ |
| Skin   | Na-K-ATPase | $0.60 \pm 0.24(10)$                                    | $0.68 \pm 0.15(9)$  | $0.51 \pm 0.08(9)$  |
|        | Mg-ATPase   | $18.66 \pm 1.39(10)$                                   | $16.54 \pm 1.24(9)$ | $15.89 \pm 0.78(9)$ |

Crabbé and DeWeer, 1964; Crabbé, 1967). Larval anurans have not been previously studied with respect to action of this hormone.

The natriferic effect of a single injection of  $1 \mu\text{g}$  aldosterone per gram body weight on larval R. catesbeiana over the period of 36 hours is shown in Figure 12. Aldosterone had no effect on Na influx or efflux for the first four hours after injection. The sodium influx of aldosterone-treated animals was significantly higher than control animals from 4 to 30 hours after injection. The peak of sodium influx of  $5.25 \pm 0.83 \mu\text{equiv}/10\text{g-hr}$  is seen from 4 to 10 hours. The sodium effluxes of aldosterone-treated and control animals, on the other hand, remain at the same level over the period of 36 hours. In all cases, hormone-treated and control animals displayed relatively high effluxes at the beginning of the experiment which probably reflects handling stress. This means that animals are in negative salt balance right after injection. Positive salt balance with a net flux of  $1.29 \pm 0.85 \mu\text{equiv}/10\text{g-hr}$  becomes apparent four hours after injection of aldosterone and persists at least 29 hours. A similar pattern has been observed in adult frogs (Maetz, 1959) and in larval Ambystoma (Alvarado and Kirschner, 1964). The latent period of aldosterone action was noted by Crabbé (1963) on isolated toad bladder and subsequently confirmed by Sharp and Leaf (1966) and Edelman and Fimognari (1968). The suggestion by Crabbé (1961a) that the latent period was the time needed to synthesize or

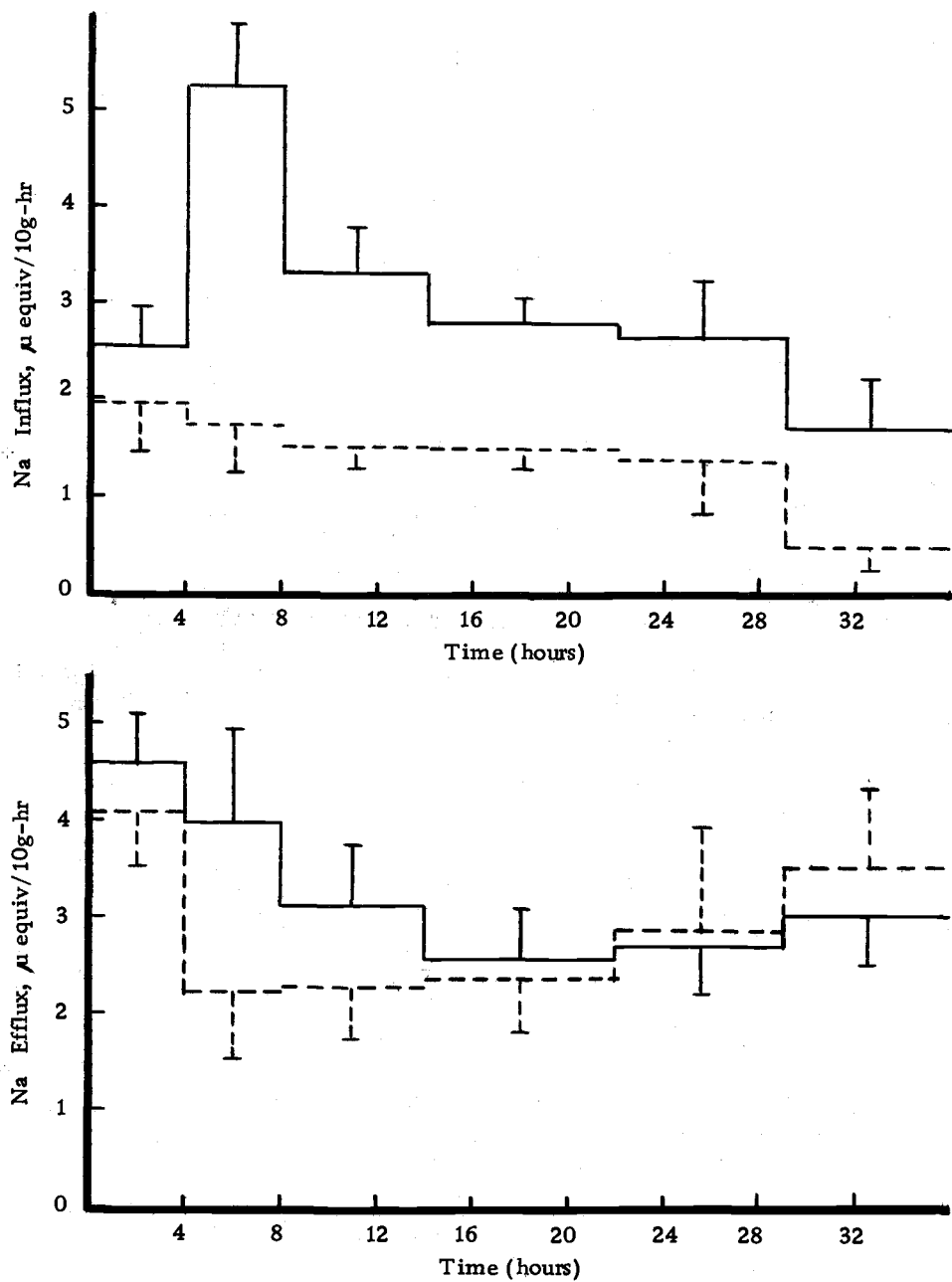


Figure 12. The effect of a single injection of aldosterone ( $1 \mu\text{g/g}$ ) on sodium fluxes in larval bullfrogs (solid line). Control animals (broken line) were injected with  $0.01 \text{ ml/g}$  body weight of the diluent ( $10\%$  ethanol in  $90 \text{ mM NaCl}$ ). The number of animals in each case was eight.



activate an intermediate involved in active sodium transport led Edelman and Fanestil (1970) to propose the induction hypothesis for the mechanism of aldosterone action.

Table 8 shows results of an experiment which was designed to compare sodium fluxes with ATPase activities in aldosterone-treated and control animals. In this experiment the fluxes were measured for 10 hours after appropriate injection. Then the animals were killed and gills and kidney were assayed for ATPase activity. As was indicated in previous experiments, aldosterone action is seen best from 4 to 10 hours after hormone injection. Values on the left side of Table 8 represent the flux values from 4 to 10 hours after appropriate injections. The flux values from zero to 4 hours, not shown in this table, are similar to those in the previous experiment. That is to say, there is no difference in sodium influx and efflux is high in both aldosterone-treated and control animals. From 4 to 10 hours aldosterone-treated tadpoles show an 83% increase of sodium influx while efflux remains constant. This results in a net flux of  $1.08 \pm 0.31 \mu\text{equiv}/10\text{g-hr}$  compared to a negative salt balance with net flux of  $-0.50 \pm 0.78 \mu\text{equiv}/10\text{g-hr}$  for control animals. The specific activities of ATPases are shown on the right side of Table 8. Gill Na-K-ATPase of aldosterone-treated animals was  $2.65 \pm 0.09 \mu\text{moles } P_i/\text{mg pr/hr}$  which was significantly higher than the activity of  $2.23 \pm 0.20 \mu\text{moles } P_i/\text{mg pr/hr}$  in control animals ( $P < 0.01$ ).

Table 8. The effect of a single intraperitoneal injection of aldosterone on sodium exchange and ATPase activities in the microsomal fractions of gill and kidney tissues of bullfrog tadpoles.

| Treatment   | Flux, $\mu$ equiv/10g-hr |       |           | Enzyme, $\mu$ moles/mg pr/hr |       |        |       |
|-------------|--------------------------|-------|-----------|------------------------------|-------|--------|-------|
|             |                          |       |           | Gill                         |       | Kidney |       |
|             | $M_i$                    | $M_o$ | $M_{net}$ | Na-K                         | Mg    | Na-K   | Mg    |
| Aldosterone | 3.52*                    | 2.44  | 1.08      | 2.65                         | 29.21 | 11.74  | 22.77 |
|             | $\pm$                    | $\pm$ | $\pm$     | $\pm$                        | $\pm$ | $\pm$  | $\pm$ |
|             | 0.38                     | 0.41  | 0.31      | 0.09                         | 0.37  | 0.74   | 1.56  |
|             | (8)                      | (8)   | (8)       | (8)                          | (8)   | (8)    | (8)   |
| Control     | 1.92                     | 2.42  | -0.50     | 2.23                         | 27.93 | 11.55  | 23.56 |
|             | $\pm$                    | $\pm$ | $\pm$     | $\pm$                        | $\pm$ | $\pm$  | $\pm$ |
|             | 0.36                     | 0.26  | 0.38      | 0.20                         | 1.46  | 0.69   | 0.82  |
|             | (7)                      | (7)   | (7)       | (7)                          | (7)   | (7)    | (7)   |

Experimental animals were injected with 0.01 ml/g of 20 mg% aldosterone. The diluent was 10% ethanol in 90 mM NaCl. Control animals received only the diluent. Flux measurements were initiated four hours after injection and lasted six hours whereupon the tissues were excised for the enzyme assay.

\*Significantly different from control animals ( $P < 0.02$ ).

This represents a 19% rise of Na-K-ATPase specific activity. Kidney Na-K-ATPase and Mg-ATPase activities are not different in the two groups.

Table 9 shows results of an experiment in which tadpoles received daily injections of 0.01 ml/g body weight of 10 mg aldosterone in 10% ethanol and 90 mM NaCl for four days. Control animals were injected daily with 0.01 ml/g body weight of diluent for four days. Sodium fluxes were measured for 10 hours after the last injection, then ATPase activities were assayed in gills and kidney. In aldosterone-treated animals sodium influx was  $3.14 \pm 0.47$   $\mu$ equiv/10g-hr compared with  $1.82 \pm 0.23$   $\mu$ equiv/10g-hr of control animals. The specific activity of gill Na-K-ATPase in aldosterone-treated animals was  $3.63 \pm 0.35$   $\mu$ moles  $P_i$ /mg pr/hr which represents a 50% increase over the control animals. The gill Mg-ATPase, on the other hand, showed no difference between these two groups of animals. Sequential injection of aldosterone also significantly increased Na-K-ATPase in the kidney with no effect on Mg-ATPase.

### Cortisol

It is quite certain that the adrenal glucocorticoids (by mammalian standards) perform a mineralocorticoid function in teleosts (Chan et al., 1968; Mayer et al., 1967). Cortisol plays a role in regulating salt-excretion in fishes living in salt water as well as regulating salt-absorption in freshwater-adapted fishes (Chan et al., 1969). Gill

Table 9. The effect of sequential injection of aldosterone on sodium exchange and ATPase activity in the microsomal fraction of gill and kidney of the bullfrog tadpole.

| Treatment   | Flux, $\mu$ equiv/10g-hr |       |           | Enzyme, $\mu$ moles/mg pr/hr |       |        |       |
|-------------|--------------------------|-------|-----------|------------------------------|-------|--------|-------|
|             |                          |       |           | Gill                         |       | Kidney |       |
|             | $M_i$                    | $M_o$ | $M_{net}$ | Na-K                         | Mg    | Na-K   | Mg    |
| Aldosterone | 3.14*                    | 6.81  | -3.67     | 3.63**                       | 24.06 | 13.01* | 21.51 |
|             | $\pm$                    | $\pm$ | $\pm$     | $\pm$                        | $\pm$ | $\pm$  | $\pm$ |
|             | 0.47                     | 0.81  | 0.54      | 0.35                         | 0.72  | 0.56   | 1.00  |
|             | (12)                     | (12)  | (12)      | (12)                         | (12)  | (12)   | (12)  |
| Control     | 1.82                     | 5.58  | -3.76     | 2.42                         | 24.95 | 10.93  | 21.13 |
|             | $\pm$                    | $\pm$ | $\pm$     | $\pm$                        | $\pm$ | $\pm$  | $\pm$ |
|             | 0.23                     | 0.76  | 0.76      | 0.16                         | 0.96  | 0.39   | 0.92  |
|             | (11)                     | (11)  | (11)      | (11)                         | (11)  | (11)   | (11)  |

Experimental animals were injected with 0.01 ml/g/day of 10 mg% d-aldosterone for four days. The diluent was 10% ethanol in 90 mM NaCl. Control animals received 0.01 ml/g/day. Flux measurements were initiated four hours after the last injection and continued for six hours whereupon tissues were excised for enzyme assay.

\*Significantly different from control animals ( $P < 0.05$ ).

\*\*( $P < 0.01$ ).

filament and kidney Na-K-ATPase activities of eel, Anguilla rostrata, and killifish, Fundulus heteroclitus, were found to be under the control of cortisol (Pickford et al., 1970; Epstein et al., 1967; Epstein et al., 1969; Epstein et al., 1971). Thus, the possible effect of cortisol on the sodium transport and gill Na-K-ATPase in amphibian larvae should be investigated.

The effect of cortisol on sodium transport in larval R. catesbiana is shown in Figure 13. There was a slight increase in sodium influx in the control animals for the first 24 hours, while the efflux remained at the same level over this period. Table 10 shows flux values and ATPase activities 10 hours after a single injection of 5  $\mu\text{g/g}$  of cortisol. Cortisol-treated animals had a sodium influx of  $2.27 \pm 0.37 \mu\text{equiv}/10\text{g-hr}$  which is not significantly higher than  $1.56 \pm 0.20 \mu\text{equiv}/10\text{g-hr}$  in control animals. Gill and kidney ATPase activities indicate no difference between these two groups of animals.

Results of sequential injections of 2  $\mu\text{g/g/day}$  of cortisol for four days are shown in Table 11. In this case, sodium influx ( $2.19 \pm 0.16 \mu\text{equiv}/10\text{g-hr}$ ) is significantly different from control animals ( $1.21 \pm 0.16 \mu\text{equiv}/10\text{g-hr}$ ;  $P < 0.05$ ). The Na-K-ATPase in the gill tissue also is significantly increased ( $2.88 \pm 0.18 \mu\text{moles } P_i/\text{mg pr/hr}$  in treated animals and  $2.37 \mu\text{moles } P_i/\text{mg pr/hr}$  for control animals). Since cortisol is not elaborated from the interrenal of bullfrogs (Mehdi and Carballeira, 1971), the negative response of

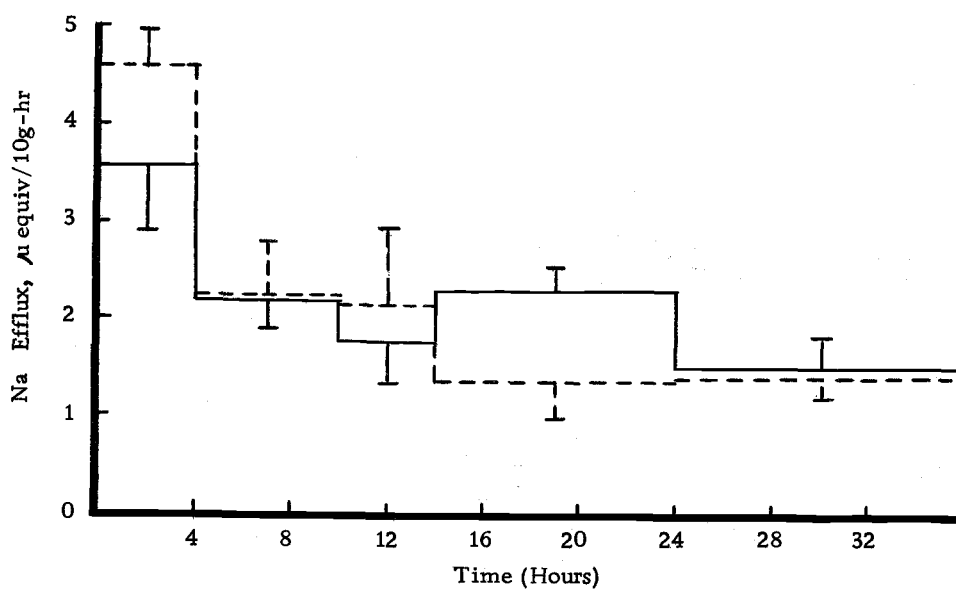
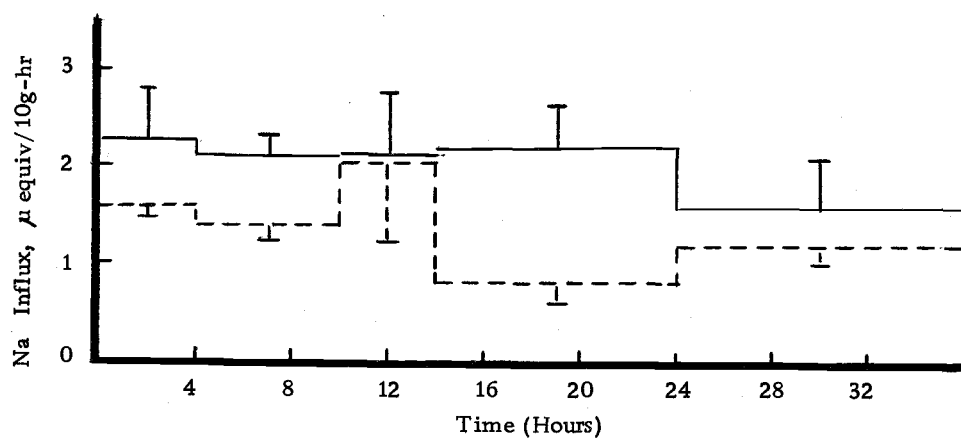


Figure 13. The effect of a single injection of 0.01 ml/g body weight of 50 mg% cortisol on sodium fluxes in larval bullfrogs (solid line). The diluent was 10% ethanol in 90 mM NaCl. Control animals (broken line) received 0.01 ml/g body weight of the diluent. (N = 8 in each case).

Table 10. The effect of a single intraperitoneal injection of cortisol on sodium exchange and ATPase activity in the microsomal fraction of gill and kidney from bullfrog tadpoles.

| Treatment | Flux, $\mu$ equiv/10g-hr |             |             | Enzyme, $\mu$ moles Pi/mg pr/hr |             |             |             |
|-----------|--------------------------|-------------|-------------|---------------------------------|-------------|-------------|-------------|
|           |                          |             |             | Gill                            |             | Kidney      |             |
|           | $M_i$                    | $M_o$       | $M_{net}$   | Na-K                            | Mg          | Na-K        | Mg          |
| Cortisol  | 2.27                     | 2.86        | -0.59       | 2.24                            | 27.50       | 12.34       | 24.31       |
|           | $\pm$                    | $\pm$       | $\pm$       | $\pm$                           | $\pm$       | $\pm$       | $\pm$       |
|           | 0.39<br>(8)              | 0.86<br>(8) | 0.52<br>(8) | 0.13<br>(8)                     | 1.70<br>(8) | 0.88<br>(8) | 0.72<br>(8) |
| Control   | 1.56                     | 2.42        | -0.86       | 2.22                            | 26.98       | 12.12       | 23.86       |
|           | $\pm$                    | $\pm$       | $\pm$       | $\pm$                           | $\pm$       | $\pm$       | $\pm$       |
|           | 0.20<br>(8)              | 0.92<br>(8) | 0.94<br>(8) | 0.20<br>(8)                     | 2.56<br>(8) | 0.42<br>(8) | 1.13<br>(8) |

The dose was 0.01 ml/g of body weight of 50 mg% cortisol in 90 mM NaCl. Control animals were injected with 0.01 ml/g of body weight of the diluent. Fluxes were measured from the fourth to the tenth hour after injection at which time tissues were excised for enzyme assay.

Table 11. The effect of sequential injection of cortisol on sodium exchange and ATPase activity in the microsomal fraction of gill and kidney tissues of bullfrog tadpoles.

| Treatment | Enzyme, $\mu\text{moles Pi/mg pr/hr}$ |             |                  |             |             |             |             |
|-----------|---------------------------------------|-------------|------------------|-------------|-------------|-------------|-------------|
|           | Flux, $\mu\text{equiv/10g-hr}$        |             |                  | Gill        |             | Kidney      |             |
|           | $M_i$                                 | $M_o$       | $M_{\text{net}}$ | Na-K        | Mg          | Na-K        | Mg          |
| Cortisol  | 2.19*                                 | 6.76        | -4.57            | 2.88*       | 20.68       | 11.43       | 20.50       |
|           | $\pm$                                 | $\pm$       | $\pm$            | $\pm$       | $\pm$       | $\pm$       | $\pm$       |
|           | 0.16<br>(8)                           | 0.78<br>(8) | 0.74<br>(8)      | 0.18<br>(8) | 0.47<br>(8) | 0.59<br>(8) | 0.60<br>(8) |
| Control   | 1.21                                  | 6.91        | -5.70            | 2.37        | 20.62       | 11.48       | 23.64       |
|           | $\pm$                                 | $\pm$       | $\pm$            | $\pm$       | $\pm$       | $\pm$       | $\pm$       |
|           | 0.16<br>(8)                           | 0.90<br>(8) | 0.81<br>(8)      | 0.28<br>(8) | 0.66<br>(8) | 0.70<br>(8) | 0.70<br>(8) |

Experimental animals received a daily intraperitoneal injection of 0.01 ml/g body weight of 20 mg% cortisol made in 90 mM NaCl for four days. Control animals received 0.01 ml/g of the diluent at the same time. Flux measurements were initiated four hours after the last injection and lasted six hours whereupon gills and kidneys were excised and assayed for ATPase.

\*Significantly different from control animals ( $P < 0.05$ ).



animals to a single injection of cortisol was not surprising. However, cortisol at a heavy dose, above physiological concentration, can induce Na transport as well as specific activity of Na-K-ATPase.

### Prolactin

A role for prolactin in osmoregulation in freshwater teleost fishes has recently been established (Ball, 1969; Olivereau and Ball, 1970; Johnson et al., 1972). Larval R. catesbeiana has the same physiological problem in maintaining plasma sodium levels above those in the environment. It is therefore of interest to determine whether the effect of prolactin found in some teleost fishes is also present in larval Amphibia.

The effect of prolactin on the sodium transport was investigated in larval R. catesbeiana and is presented in Figure 14. Sodium fluxes were measured over a period of 36 hours after 25  $\mu\text{g/g}$  prolactin injection. Prolactin had no effect on sodium transport.

Sodium fluxes and ATPase activities after prolactin treatment are shown in Tables 12 and 13. There were no changes in either sodium fluxes or ATPase activities in gill and kidney after single injection of 25  $\mu\text{g/g}$  prolactin (Table 12). Sequential injections of 10  $\mu\text{g/g}$  prolactin for four days (Table 13) indicated no differences in sodium influx and ATPase activities. However, there is a significant increase in sodium retention as a result of a smaller sodium efflux ( $5.45 \pm 0.92$  compared to  $8.25 \pm 0.67$   $\mu\text{equiv/10g-hr}$  control).

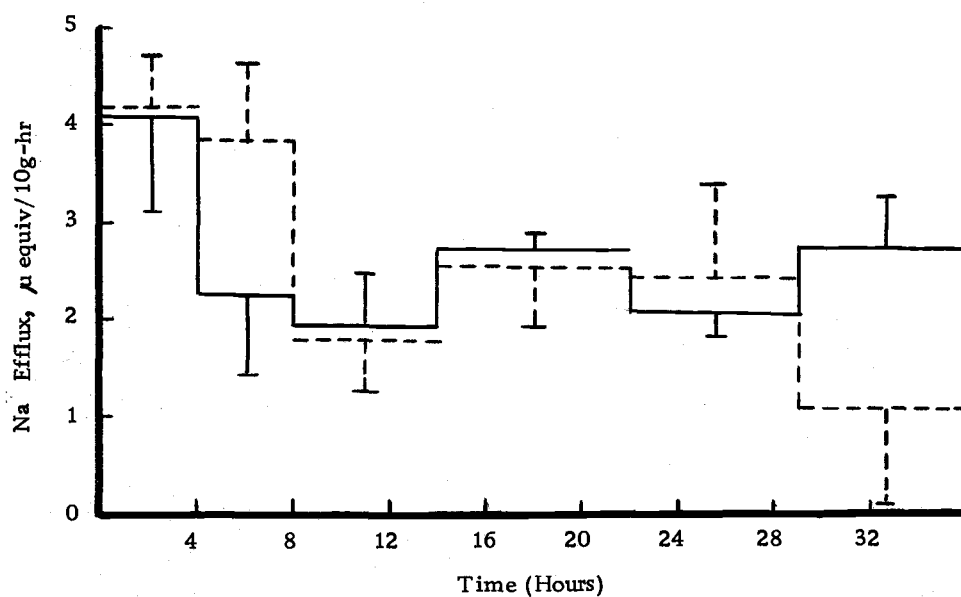
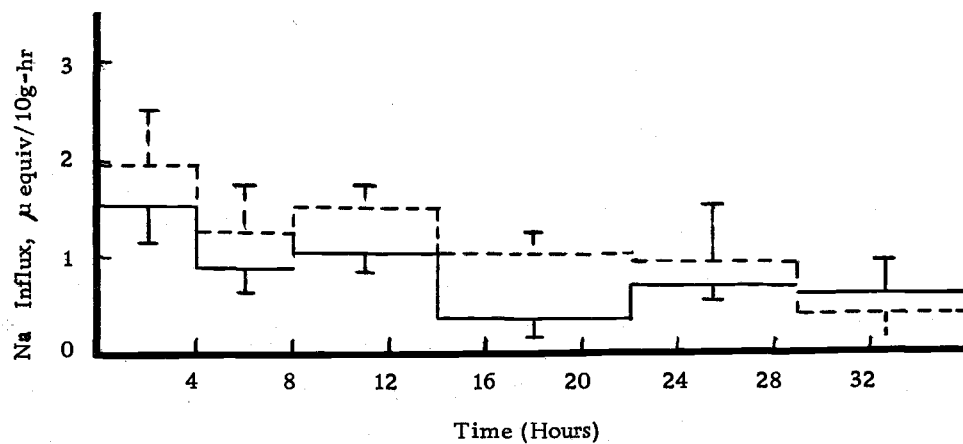


Figure 14. The effect of a single injection of 0.01 ml/g body weight of 250 mg% prolactin on sodium fluxes in larval bullfrogs (solid line). The diluent was 90 mM NaCl. Control animals (broken line) received 0.01 ml/g body weight of the diluent. (N = 8 in each case).

Table 12. The effect of a single intraperitoneal injection of prolactin on sodium exchange and ATPase activity in gill and kidney microsomal fractions from bullfrog tadpoles.

| Treatment | Flux, $\mu$ equiv/10-hr |       |           | Enzyme, $\mu$ moles $P_i$ /mg pr/hr |       |        |       |
|-----------|-------------------------|-------|-----------|-------------------------------------|-------|--------|-------|
|           | $M_i$                   | $M_o$ | $M_{net}$ | Gill                                |       | Kidney |       |
|           |                         |       |           | Na-K                                | Mg    | Na-K   | Mg    |
| Prolactin | 2.11                    | 2.33  | -0.23     | 2.49                                | 20.41 | 12.21  | 22.64 |
|           | $\pm$                   | $\pm$ | $\pm$     | $\pm$                               | $\pm$ | $\pm$  | $\pm$ |
|           | 0.51                    | 0.48  | 0.49      | 0.31                                | 0.88  | 0.54   | 1.02  |
|           | (8)                     | (8)   | (8)       | (8)                                 | (8)   | (8)    | (8)   |
| Control   | 1.43                    | 3.28  | -1.85     | 2.43                                | 21.53 | 11.93  | 22.80 |
|           | $\pm$                   | $\pm$ | $\pm$     | $\pm$                               | $\pm$ | $\pm$  | $\pm$ |
|           | 0.16                    | 0.76  | 0.70      | 0.20                                | 0.79  | 1.06   | 1.23  |
|           | (8)                     | (8)   | (8)       | (8)                                 | (8)   | (8)    | (8)   |

The dose was 25  $\mu$ g of prolactin/g. The diluent was 90 mM NaCl. Control animals were injected with 0.01 ml/g of 90 mM NaCl. Fluxes were measured from four to ten hours after injection whereupon tissues were excised for enzyme assay.

Table 13. The effect of sequential injection of prolactin on sodium exchange and ATPase activity in the microsomal fraction of gill and kidney tissues of larval bullfrogs.

| Treatment | Flux, $\mu\text{equiv}/10\text{g-hr}$ |             |                  | Enzyme, $\mu\text{moles P}_i/\text{mg pr/hr}$ |             |             |             |
|-----------|---------------------------------------|-------------|------------------|---|-------------|-------------|-------------|
|           | $M_i$                                 | $M_o$       | $M_{\text{net}}$ | Gill  |             | Kidney      |             |
|           |                                       |             |                  | Na-K  | Mg          | Na-K        | Mg          |
| Prolactin | 1.84                                  | 5.45*       | -3.61**          | 2.42  | 21.44       | 8.98        | 22.54       |
|           | $\pm$                                 | $\pm$       | $\pm$            | $\pm$   | $\pm$       | $\pm$       | $\pm$       |
|           | 0.35<br>(8)                           | 0.92<br>(8) | 1.04<br>(8)      | 0.12<br>(8)                                   | 0.72<br>(8) | 0.70<br>(8) | 9.97<br>(8) |
| Control   | 1.30                                  | 8.25        | -6.95            | 2.48  | 22.12       | 9.62        | 23.47       |
|           | $\pm$                                 | $\pm$       | $\pm$            | $\pm$   | $\pm$       | $\pm$       | $\pm$       |
|           | 0.17<br>(8)                           | 0.67<br>(8) | 0.58<br>(8)      | 0.11<br>(8)                                   | 0.80<br>(8) | 0.63<br>(8) | 0.88<br>(8) |

The dose was 10  $\mu\text{g}$  of prolactin/g per day for four days (0.01 ml/g). Control animals were injected with 0.01 ml of 90 mM NaCl each day. Flux measurements were initiated four hours after the last injection and ran six hours at which time tissues were excised for enzyme assay.

\*Significantly different from control animals ( $P < 0.05$ ).

\*\*( $P < 0.02$ ).

Prolactin seems to have no effect on sodium influx and gill and kidney ATPase activities in larval R. catesbeiana. Prolactin was found, also to have no effect on plasma sodium levels in adult R. pipiens as well as of larval R. catesbeiana, and Ambystoma tigrinum (Crim, 1972). Johnson et al. (1972) demonstrated the effects of prolactin on the urinary bladder of starry flounder (Platichthys stellatus) on contributing to hydromineral balance in this animal by modifying urine concentration. Sodium retention after prolactin treatment in larval R. catesbeiana needs further investigation.

#### Prolactin Plus Cortisol

Prolactin plus cortisol was shown to restore normal electrolyte balance of hypophysectomized eels (Anguilla anguilla) (Chan et al., 1968). Single injections of prolactin or cortisol alone in physiological doses did not have any effect on Na transport in tadpoles. There was no previous information concerning the possibility of a synergistic effect of these two hormones in amphibian larvae. This was investigated and the results are presented in Figure 15. The sodium influx after injection of 5  $\mu\text{g/g}$  of cortisol and 25  $\mu\text{g/g}$  of prolactin was significantly higher than in control animals over a period of 36 hours, while the efflux remained at the same level. Sodium fluxes and ATPase activities after single injection of 5  $\mu\text{g/g}$  cortisol and 25  $\mu\text{g/g}$  prolactin are shown in Table 14 and after sequential injections of 2  $\mu\text{g/g}$  cortisol and 10  $\mu\text{g/g}$  prolactin in Table 15. In both cases

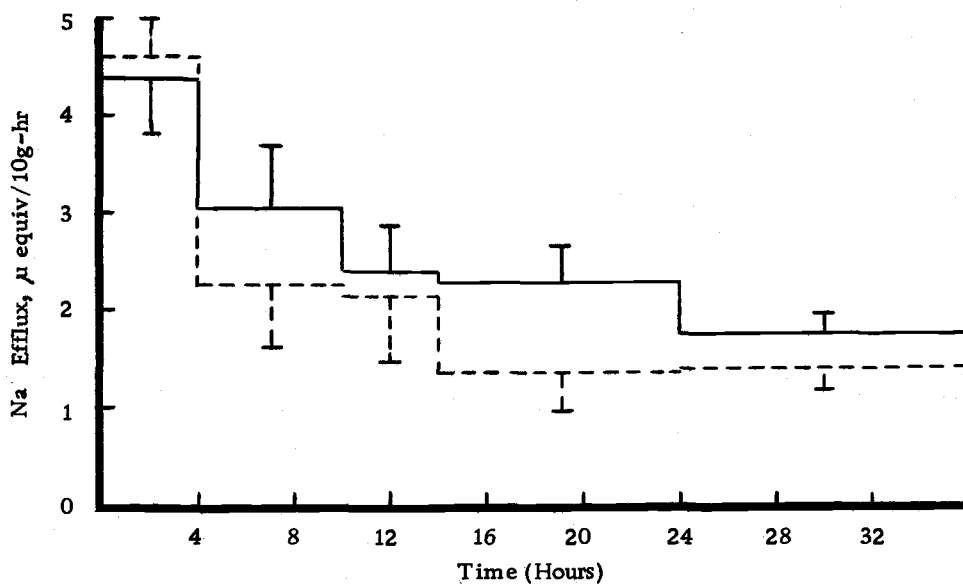
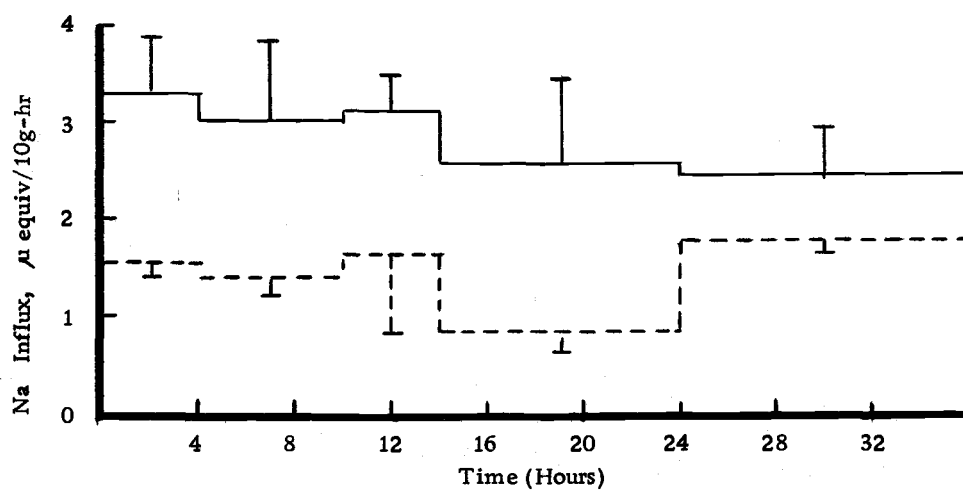


Figure 15. The effect of a single injection of 0.01 ml/g body weight of 50 mg% cortisol plus 250 mg% prolactin on sodium fluxes in larval bullfrogs (solid line). The diluent was 90 mM NaCl. Control animals (broken line) received 0.01 ml/g body weight of the diluent. (N = 8 in each case).

Table 14. The effect of a single intraperitoneal injection of cortisol plus prolactin on sodium exchange and ATPase activity in gill and kidney microsomal fractions from bullfrog tadpoles.

| Treatment               | Flux, $\mu$ equiv/10g-hr      |                              |                               | Enzyme, $\mu$ moles $P_i$ /mg pr/hr |                               |                               |                               |
|-------------------------|-------------------------------|------------------------------|-------------------------------|-------------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                         | $M_i$                         | $M_o$                        | $M_{net}$                     | Gill                                |                               | Kidney                        |                               |
|                         |                               |                              |                               | Na-K                                | Mg                            | Na-K                          | Mg                            |
| Prolactin +<br>Cortisol | 3.93*<br>$\pm$<br>0.67<br>(8) | 3.61<br>$\pm$<br>0.37<br>(8) | 0.32<br>$\pm$<br>0.57<br>(8)  | 2.62**<br>$\pm$<br>0.11<br>(8)      | 26.94<br>$\pm$<br>0.54<br>(8) | 12.02<br>$\pm$<br>1.03<br>(8) | 23.46<br>$\pm$<br>0.96<br>(8) |
| Control                 | 1.62<br>$\pm$<br>0.56<br>(7)  | 3.21<br>$\pm$<br>0.93<br>(7) | -1.59<br>$\pm$<br>0.46<br>(7) | 2.22<br>$\pm$<br>0.05<br>(7)        | 26.94<br>$\pm$<br>1.57<br>(7) | 13.12<br>$\pm$<br>1.72<br>(7) | 23.07<br>$\pm$<br>1.42<br>(7) |

The dose was 5  $\mu$ g cortisol and 25  $\mu$ g prolactin/g. The diluent was 90 mM NaCl. Control animals were injected with 0.01 ml/g of 90 mM NaCl. Fluxes were measured from four to ten hours after injection whereupon tissue were excised for enzyme assay.

\*Significantly different from control animals ( $P < 0.02$ ).

\*\*( $P < 0.01$ ).

Table 15. The effect of sequential injection of cortisol plus prolactin on sodium exchange and ATPase activity in the microsomal fraction of gill and kidney tissues of larval bullfrogs.

| Treatment               | Flux, $\mu\text{equiv}/10\text{g}\cdot\text{hr}$ |             |                  | Enzyme, $\mu\text{moles P}_i/\text{mg pr/hr}$ |             |             |             |
|-------------------------|--|-------------|------------------|---|-------------|-------------|-------------|
|                         | $M_i$  | $M_o$       | $M_{\text{net}}$ | Gill  |             | Kidney      |             |
|                         |  |             |                  | Na-K  | Mg          | Na-K        | Mg          |
| Cortisol +<br>Prolactin | 3.18*  | 4.18*       | -1.00*           | 3.36*   | 21.43*      | 9.56        | 19.26       |
|                         | $\pm$  | $\pm$       | $\pm$            | $\pm$   | $\pm$       | $\pm$       | $\pm$       |
|                         | 0.29<br>(8)                                      | 0.32<br>(8) | 0.49<br>(8)      | 0.19<br>(8)                                   | 0.83<br>(8) | 0.34<br>(8) | 0.48<br>(8) |
| Control                 | 1.75   | 8.46        | -6.71            | 2.42  | 22.42       | 10.43       | 21.14       |
|                         | $\pm$  | $\pm$       | $\pm$            | $\pm$   | $\pm$       | $\pm$       | $\pm$       |
|                         | 0.14<br>(8)                                      | 0.43<br>(8) | 0.45<br>(8)      | 0.36<br>(8)                                   | 1.27<br>(8) | 0.93<br>(8) | 0.76<br>(8) |

The dose was 2  $\mu\text{g}$  cortisol and 10  $\mu\text{g}$  prolactin/g per day for four days (0.01 ml/g). Control animals were injected with 0.01 ml of 90 mM NaCl each day. Flux measurements were initiated four hours after the last injection and ran six hours at which time tissues were excised for enzyme assay.

\*Significantly different from control animals ( $P < 0.01$ ).



injection of cortisol plus prolactin elevated both active Na transport and the specific activity of Na-K-ATPase.

### Thyroxin

The various aspects of thyroxin in relation to amphibian metamorphosis have been extensively explored (Etkin, 1970; Weber, 1967). Only a few lines of evidence indicate a possible role of thyroxin in the regulation of Na transport in amphibians. Taylor and Barker (1965) by exposing tadpoles to a low concentration of thyroxin could accelerate the appearance of the transepithelial potential difference. Thus, the possible effects of thyroxin on Na transport should be reinvestigated. Figure 16 shows Na influx and efflux respectively of tadpoles after injection of 1  $\mu$ g/g thyroxin. The fluxes of thyroxin-treated animals and control animals are not different. Table 16 shows Na fluxes and ATPase activities. Again, no sign of thyroxin effects was observed.

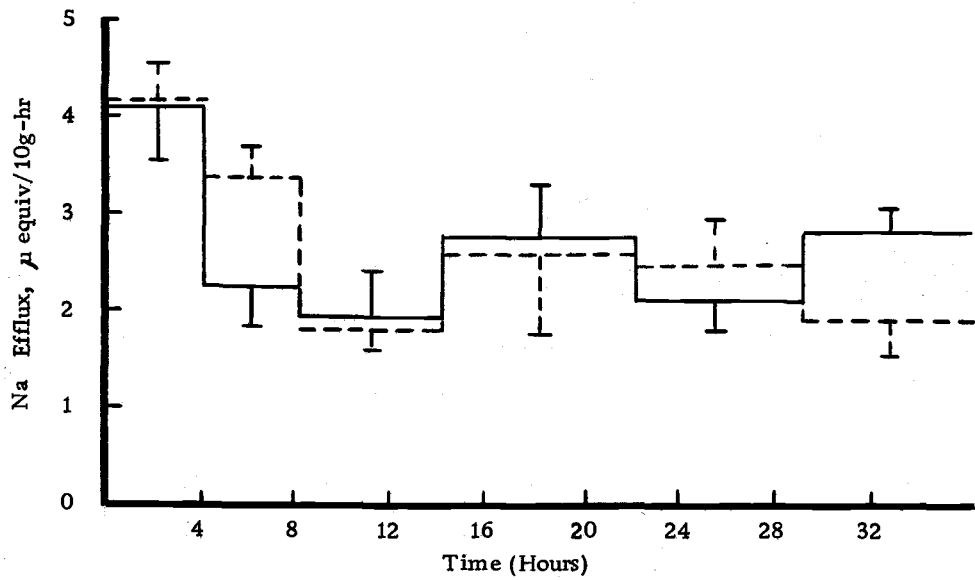
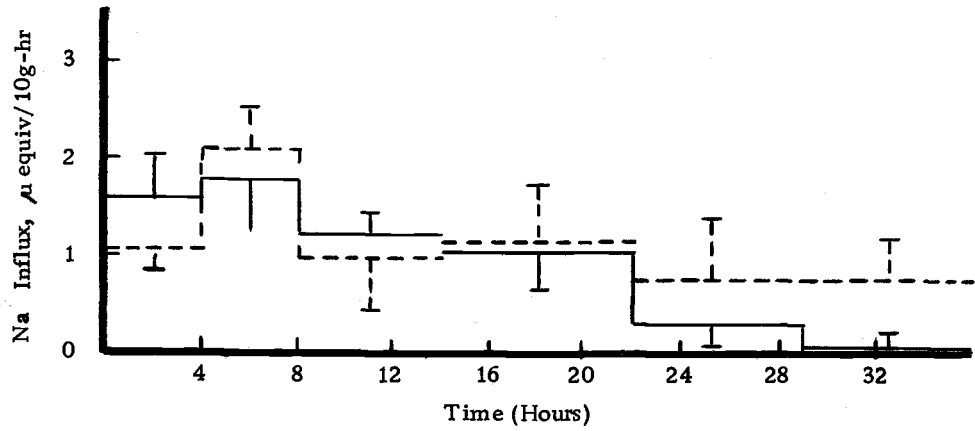


Figure 16. The effect of a single injection of 0.01 ml/g body weight of 10 mg% thyroxin on sodium fluxes in larval bullfrogs (solid line). The diluent was 90 mM NaCl. Control animals (broken line) received 0.01 ml/g body weight of the diluent. (N = 8 in each case).

Table 16. The effect of a single intraperitoneal injection of thyroxin on sodium exchange and ATPase activity in gill and kidney microsomal fractions from bullfrog tadpoles.

| Treatment | Flux, $\mu$ equiv/10g-hr |       |           | Enzyme, $\mu$ moles $P_i$ /mg pr/hr |       |        |       |
|-----------|--------------------------|-------|-----------|-------------------------------------|-------|--------|-------|
|           |                          |       |           | Gill                                |       | Kidney |       |
|           | $M_i$                    | $M_o$ | $M_{net}$ | Na-K                                | Mg    | Na-K   | Mg    |
| Thyroxin  | 1.44                     | 3.00  | -1.56     | 2.27                                | 26.23 | 12.49  | 22.95 |
|           | $\pm$                    | $\pm$ | $\pm$     | $\pm$                               | $\pm$ | $\pm$  | $\pm$ |
|           | 0.12                     | 0.60  | 0.48      | 0.24                                | 1.36  | 0.87   | 1.23  |
|           | (8)                      | (8)   | (8)       | (8)                                 | (8)   | (8)    | (8)   |
| Control   | 1.07                     | 2.29  | -1.22     | 2.22                                | 25.87 | 12.07  | 21.79 |
|           | $\pm$                    | $\pm$ | $\pm$     | $\pm$                               | $\pm$ | $\pm$  | $\pm$ |
|           | 0.21                     | 0.06  | 0.19      | 0.18                                | 1.24  | 1.34   | 1.53  |
|           | (8)                      | (8)   | (8)       | (8)                                 | (8)   | (8)    | (8)   |

The dose was 1  $\mu$ g of thyroxin/g. The diluent was 90 mM NaCl. Control animals were injected with 0.01 ml/g of 90 mM NaCl. Fluxes were measured from four to ten hours after injection whereupon tissues were excised for enzyme assay.

## DISCUSSION

The transport of Na across cell membranes against an electrochemical gradient requires energy. This energy is derived from the hydrolysis of ATP (Caldwell et al., 1960; Whittam and Ager, 1965; Davis et al., 1964). Transport-coupled hydrolysis of ATP in animal cells is mediated in part by a relative specific ATPase which requires Na and K as well as Mg for maximum activity (Skou, 1957, 1964). It is well documented that Na-K-ATPase is intimately involved in active Na transport (Skou, 1965; Katz and Epstein, 1968; Bonting, 1970). The enzyme system has been found in a variety of transport epithelia including skin of adult frogs (Bonting and Caravaggio, 1963; Kawada et al., 1969), toad urinary bladder (Bonting and Caravaggio, 1963), turtle urinary bladder (Shamoo and Brodsky, 1970), gills and kidney of euryhaline fishes (Pickford et al., 1970), and gills of freshwater fishes (Epstein et al., 1967; Kamiya and Utida, 1969).

The gills of tadpoles may be involved in transepithelial ion transport (Alvarado and Moody, 1970). Gills of fishes and tadpoles have similar origin but probably are not truly homologous (Schmathausen, 1968). In any event, there may be a functional similarity with respect to their role in ion absorption.

Subcellular localization of tadpole gill Na-K-ATPase indicates its highest activity in the microsomal fraction. The microsomal

fraction was confirmed to be broken cellular membranes (Hanzon and Toshi, 1959; Wallach and Kamat, 1964; Kamat and Wallach, 1965).

The Na-K-ATPase from tadpole gill tissue generally displays similar characteristics to the enzyme from other tissues in the requirement for ATP as a substrate, Na and K activation, and the inhibition by ouabain (Bonting and Caravaggio, 1963; Matsui and Schwartz, 1966; Shamoo and Brodsky, 1970). The values of pH optimum, temperature optimum and  $Q_{10}$ , and of the  $K_m$  for Na and K but not the  $K_m$  of ATP were similar to corresponding values reported for other tissues (Skou, 1962; Matsui and Schwartz, 1966; Shamoo and Brodsky, 1970).

The affinity of the enzyme for ATP in tadpole gill was lower ( $K_m = 0.6$ ) than has been reported for turtle bladder ( $K_m = 0.25$ ) or fish gills (Motais, 1972; Shamoo and Brodsky, 1970). Removal of Na and/or K from the reaction mixture completely inhibited the ouabain sensitive components of the ATPase activity in gill tissue.

The Na-K-ATPase activity in the tadpole gills was higher than other tadpole tissues examined except the kidney. It was even higher than in the skin of adult bullfrogs which is known to be involved in transepithelial transport. The Na-K-ATPase activity in the skin of larvae and adults was the same as reported by Kawada *et al.* (1969). There was an inverse correlation of Na-K-ATPase activity in gills and skin of tadpoles. At the early stage of metamorphosis (stage X to XVIII) there is a high activity in gills and low activity in skin. At

later stages, gill activity is decreased and disappears at stage XXV, while the skin enzyme activity is tripled. This indicates a shift of the route of active Na transport from gills to skin during metamorphosis.

Salt depletion stimulates active Na absorption and causes a highly significant increase in gill Na-K-ATPase. Salt loading, on the other hand, reduces the gill Na-K-ATPase activity and the active absorption of Na is also depressed. The inverse relationship of Na-K-ATPase with external salt concentration reveals that tadpoles seem to be able to adjust and control the specific activity of Na-K-ATPase according to physiological need. The mechanisms for salt depletion and salt loading responses remain unknown. However the relatively slow time course of responses suggests endocrine mediation. Hormones from the hypothalamic neurohypophysial complex, the anterior pituitary and the interrenal tissue have been implicated in hydro-mineral metabolism (Alvarado and Johnson, 1966; Crabbé, 1963). Salt depletion elevates and salt loading depresses aldosterone levels in toad blood (Crabbé, 1963).

Even though it has been demonstrated beyond a reasonable doubt that aldosterone at physiological concentrations increases the active transport of sodium, there is disagreement on the basic mechanism. There are three schools of thought; the first school led by I. S. Edelman (Edelman and Fanestil, 1970) states that aldosterone stimulates

Na transport by increasing the activity of one or several metabolic steps resulting in increased ATP for the sodium pump; the second school led by G. W. Sharp and A. Leaf (1963) believe that aldosterone acts by increasing permeability of apical or mucosal surface of epithelial membrane to sodium; the third school believes that aldosterone may act by stimulating the production of the active pump or its equivalent enzyme Na-K-ATPase.

Isolated toad urinary bladders have been widely used to study the effect and mode of action of aldosterone (Bonting and Canady, 1964; Crabbé, 1961a, 1961b; Crabbé and DeWeer, 1964; Sharp and Leaf, 1963; Crabbé and Ehlich, 1968; Fanestil, et al., 1968; Edelman and Fimognari, 1968; Leaf and Sharp, 1971). In this preparation aldosterone effects sodium movement by increasing the active sodium transport without affecting the specific activity of Na-K-ATPase. Similar results have been reported for rat kidney (Chignell and Titus, 1966; Landon et al., 1966). On the other hand, adrenalectomy of rats is followed by a gradual decline in renal Na-K-ATPase activity which can be countered by injection of aldosterone (Jorgensen, 1972). In the eel cortisol acts as a mineralocorticoid and stimulates branchial Na-K-ATPase activity (Epstein et al., 1971).

This study clearly shows that aldosterone increases the specific activity of Na-K-ATPase in gills and, when injected sequentially, in kidneys. There is little question that the sodium pump is the major

target for this hormone and there is increasing evidence for the identity of the pump and the Na-K-ATPase system. This is not to imply, necessarily, a direct action of the hormone on the synthesis or modulation of the activity of the Na-K-ATPase already present in the tissues. This would require more detailed studies, preferably on an in vitro system.

Cortisol (17-hydroxycorticosterone), like aldosterone, is a steroid derived from the adrenal cortex. They are similar in structure, the difference being that aldosterone has an aldehyde at carbon 12 instead of a methyl group and a hydroxyl on carbon 17. The aldehyde permits aldosterone to exist in a hemiacetyl form. In higher vertebrates the function of the two steroids differ markedly. Cortisol is a glucocorticoid with little effect on Na metabolism whereas aldosterone has little glycogenic activity and plays a major role in ion balance. Studies on the lower vertebrates indicate that the distinction is not as clear as in higher forms. Cortisol is synthesized by adrenal cortical tissue of eels (Sandor et al. , 1966). Cortisol as well as aldosterone is able to restore the plasma sodium levels due to the effects of adrenalectomy in eels (Henderson and Chester Jones, 1967). Cortisol increases the specific activity of Na-K-ATPase in osmoregulation-participating organs--gills, kidney and intestine of fresh-water eel (Anguilla rostrata) (Epstein, et al. , 1971).



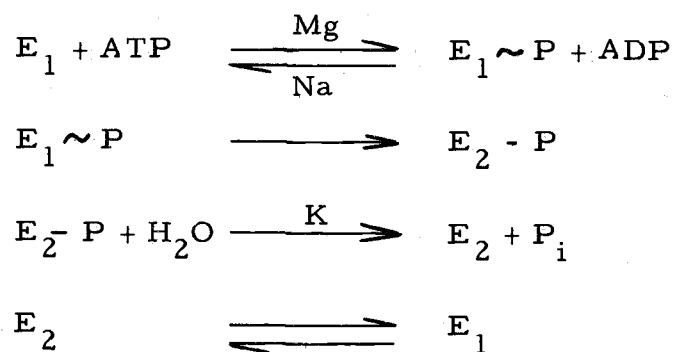
A different pattern of cortisol response is observed in bullfrog tadpoles. Sequential treatment elevated the specific activity of Na-K-ATPase and the sodium influx while a single injection did not. This probably reflects a pharmacological effect of high levels of cortisol. The characteristic decrease in plasma sodium concentration following hypophysectomy in some teleosts can be corrected by prolactin. Generally prolactin maintains plasma sodium levels by increasing sodium retention. However, there are conflicting results on the effects of prolactin on sodium-retaining activity in amphibians. No effect was observed on hypophysectomized leopard frog R. pipiens and California newts Taricha torosa (Crim, 1972), on the other hand it could reduce Na loss in larval Ambystoma mexicanum (Whittouck, 1972). The present investigation indicates no effect of prolactin on sodium uptake in intact bullfrog larvae while retention of sodium is clearly evident. There was no effect on Na-K-ATPase, suggesting that this hormone may decrease passive permeability of exposed epithelia to Na.

The mechanism of synergistic action of cortisol and prolactin on the specific activity of Na-K-ATPase and the sodium fluxes is unknown. There is no latent period for the effect of these two hormones. Cortisol alone has only a small effect on this animal as indicated in previous experiments; while prolactin alone can increase sodium retention. It seems likely that the synergistic effect of these

two hormones might be just simply adding the effects of each hormone.

The cation transport system utilizes energy derived from hydrolysis of ATP. Skou (1957) pointed out that an ATPase would be required to make energy available for this transport. The activity of ATPase isolated from crab nerve was increased considerably by adding Na and K to the assay medium and this additional ATPase activity was completely inhibited by ouabain. Ouabain had been shown earlier to be specific inhibitor of active transport in erythrocytes (Schatzmann, 1953). Therefore, Skou (1957) suggested that this ouabain-sensitive Na-K-activated ATPase might be part of, or identical with, the cation transport system. Both the transport system and Na-K-ATPase should have similar characteristics with regard to the activation by Na and K, utilization of ATP, and ouabain inhibition. The enzyme system should also have the following characteristics: it should be associated with cell membranes; it should have an affinity for Na that is higher than for K on the inside of the cell membrane and the reverse for the outside; it should be found in all cells in which an active transport occurs. A great number of reports on the properties of Na-K-ATPase from various tissues confirmed these similarities. Recent work on Na-K-ATPase has dealt with enzyme purification and the mechanism of enzyme reaction.

The ionic specificity of the enzyme is thought to change upon phosphorylation and dephosphorylation. The sequence, as envisioned by Siegel and Albers (1967), is as follows:



Various phosphorylated intermediates have been identified (Uesugi, 1971; Robinson, 1971). Presumably  $E_1$  and  $E_1 \sim \text{P}$  would have a high affinity for Na and this form would predominate on the inner surface of the cell membrane whereas  $E_2 - \text{P}$  and  $E_2$  would predominate on the outer surface and have a low affinity for Na. The reverse would be true for K.

The transport of Na from a very dilute bath into body fluids across a layer of cells like the gills involves transfer across two successive membranes. Transport across the inner membrane is essentially equivalent to Na extrusion, which is characteristic of practically all animal cells. It requires Na-K-ATPase and occurs against both a concentration and an electrical gradient (the inside of most cells is negative to the outside by 50-100 mV). Part of the problem of transepithelial transport then could be solved by

localizing the "pump" on the inner membrane. The problem then is how to get Na from the bath into the transport pool in the cell. Since Na uptake occurs from very dilute solutions it seems unlikely that the electrical gradient alone would suffice and even less likely that the Na concentration in the cell could be reduced to a value which would allow for simple diffusion of Na into the cell. There is increasing evidence that there is a separate "pump" at the outer membrane, the nature of which remains unknown (Maetz, 1971; Motais and Garcia Romeu, 1972; Alvarado and Dietz, 1970).

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