THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

OSMOTIC AND IONIC REGULATION IN THE RED RIVER PUPFISH, *CYPRINODON RUBROFLUVIATILIS*

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JAMES LARRY RENFRO

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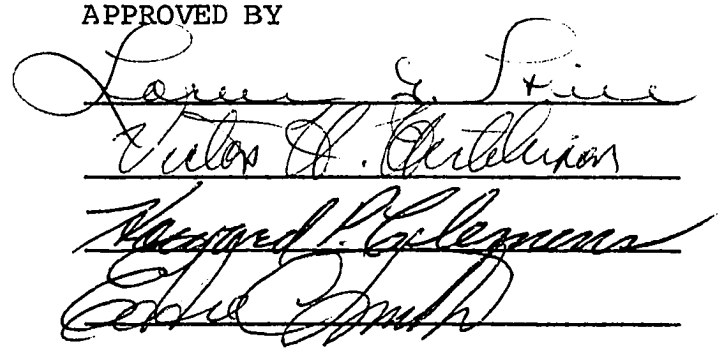
OSMOTIC AND IONIC REGULATION IN THE RED RIVER PUPFISH, CYPRINODON RUBROFLUVIATILIS

APPROVED BY



DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Homer Smith (1930) showed that saltwater-acclimated fishes swallowed their surrounding medium and subsequently absorbed most of the monovalent ions and water through the gut wall. A large part of the water and salts gained in this way were excreted extrarenally as a solution hyperosmotic to the surrounding medium. A small fraction of the water was then used to form urine which was isosmotic or hypoosmotic to the blood. Smith (1930) also reported that drinking did not occur in freshwater and supposed that water and salt gain in this medium was through the oral and gill membranes.

The original model set forth by Smith (1930) for osmoregulation in fishes has undergone only slight modification since its inception. We now know that freshwater fishes also drink their surrounding medium and that even in saltwater-acclimated fishes, drinking accounts for only a small portion of the total salt and water turnovers (Motais and

Maetz, 1965; Dall and Milward, 1969 ; Potts and Evans, 1967; Maetz and Skadhauge, 1968; Lotan, 1969). For both freshwater- and saltwater-acclimated fishes, the larger portions of salts and water enter and exit via the gills (Fromm, 1968; Richards and Fromm, 1970; Motais, et al, 1969).

Lotan (1969) showed that the most important mode of osmoregulation in Aphanius dispar (Cyprinodontidae) was an alteration in total body sodium and chloride content with changing salinity. Potts and Evans (1967) and Stanley and Fleming (1966) reported large changes in the total body sodium content of two species of Fundulus (Cyprinodontidae) when the external salinity was altered. Following Motais (1967) these fishes would be considered "osmoconformers". By his reasoning osmoconformers exhibit internal osmotic pressure changes that directly follow changes in the external medium. Fishes such as the european eel, Anguilla (Motais, Garcia Romeu, and Maetz, 1966) and the mouth-brooder, Tilapia (Potts, et al, 1967), which undergo very little change in total ion content when the ambient salinity changes, would be considered "osmoregulators" by Motais (1967). The difference in the two terms used is one of degree since all teleosts studied have been shown capable, if fully acclimated, of maintaining relative constancy of their internal medium.

Several species of the family Cyprinodontidae have been studied with regard to osmoregulation (F. heteroclitus: Potts and Evans, 1967; and Motais, et al, 1966; F. kansae: Stanley and Fleming, 1966; A. dispar: Lotan, 1969). Perhaps no genus of this family has shown more tolerance to salinity than the type genus, Cyprinodon. Several species of Cyprinodon have been found in natural waters three to five times more saline than sea water (Barlow, 1958; Simpson and Gunter, 1956). They seem to survive equally well in freshwater, however, and the distribution of certain forms appears to be independent of ionic concentrations and of the ratios of various ionic combinations (Martin, 1968).

The Red River pupfish, C. rubrofluviatilis, inhabits the shallow waters of the western drainages of the Brazos and Red rivers in Texas and Oklahoma. Echelle (1970) found this species in waters ranging from 0.42 to 51.7 parts per thousand dissolved solids. L. G. Hill (personal communication) has maintained pupfish in the laboratory in 9% NaCl, and I have found the fish abundant in natural waters of 5% NaCl.

The purposes of this study were to examine the ability of this fish to osmoregulate and to identify some of the mechanisms involved.

CHAPTER II

METHODS AND MATERIALS

Animals and Media

Adult C. rubrofluviatilis, weighing 0.5 to 2.0 g, were taken by seine from the Prairie Dog Town Fork of the Red River near Hollis, Oklahoma, and Childress, Texas. After transportation to the Norman campus of the University of Oklahoma in styrofoam ice chests, all fish were maintained in aged tap water in 45-gallon aquaria. The aged tap water was continually refiltered through glass wool. The aquaria were located in a controlled temperature room at 21 C with an automatically regulated light-dark cycle (12 hours light: 12 hours dark). Each aquarium accommodated a maximum of 150 to 200 fish (ca. one fish per liter). The daily diet of the fish consisted of commercial fish food (Tetra-Min) and washed brine shrimp. Food was always withheld 24 hours before experimentation.

The animals were acclimated to two osmotic and ionic situations. Freshwater (hypoosmotic medium) -acclimated

fish had been maintained as above for two weeks. The osmotic pressure of this medium ranged from 65 to 75 m-osmoles per Kg-water (expressed hereafter simply as m-osmoles) with a sodium and potassium content of 42 mEq/l (milliequivalents per liter) and 5 mEq/l, respectively. Saltwater (hyperosmotic medium) -acclimated fish were those which, after the routine two week laboratory acclimation to freshwater, had been maintained for 2-21 days in aged, conditioned tap water to which sufficient reagent grade sodium chloride (Merke or Fisher) had been added to raise the osmotic pressure to a range of 966 to 1130 m-osmoles. This hyperosmotic medium had an average sodium and potassium content of 424 mEq/l and 5 mEq/l, respectively.

Collection and Preparation of Blood Samples

Blood samples were obtained by blind cardiac puncture. The punctures were made with non-heparinized coagulation capillary tubes (1.3-1.5 mm, i.d.) heated and drawn to fine tipped pipettes. About 30-50 ul of blood were collected from each animal. Fish were retrieved by netting, immediately blotted dry, and the body wall and heart punctured with a capillary pipette. The naked breast of this species facilitated the cardiac puncture. The blood filled the pipette quickly without application of suction. In some

instances, slight contamination of the blood resulted from fluid in the pericardial cavity. This was ignored except in the case of the inulin space experiments where samples visibly contaminated were discarded. Hemolyzed samples were discarded in all experiments. The blood-filled capillary pipettes were centrifuged in a clinical centrifuge (International Equipment Company) equipped with an hematocrit head at 7500 rpm for a sufficient time to pack the cells. All tests were conducted on the supernatant. This supernatant will be referred to as serum; however, no attempt was made to determine whether it was plasma or serum. After centrifugation, the collection pipettes were broken, and Beckman micropipettes were used to withdraw the serum.

Serum Osmotic Pressure

Vapor pressure osmometry (Mechrolab, Model 301A) was used to measure serum osmotic pressures. Due to the small amount of serum obtained from each fish, the usual microtechnique employed with this instrument was not adequate. A special technique was used in this study (H.B. Haines, personal communication). This technique substituted #2 flint glass tubing approximately 12 inches long, heated and drawn to a fine tipped micropipette for the usual 50-ul microsyringe.

The thermisitor bead was washed thoroughly with deionized, distilled water. The final drop of distilled water was drawn off by use of one of the large sample syringes. A drop of the serum sample was then placed on the thermisitor bead and allowed to remain a few seconds. This drop was then drawn off with a large syringe, and the step repeated. In all cases sample removal left the bead clear. A reading was taken on the next drop of sample and compared to NaCl standards. This technique eliminated the large sample volumes normally needed to rinse the bead; it also made sample delivery more accurate and the drop size smaller; therefore, the serum sample was conserved. This method appeared to yield better precision than the usual micro-technique. It also eliminated the necessity of pooling samples so that each reading represented one fish.

The serum osmotic pressure was examined upon transfer of fish from the freshwater medium to the saltwater medium described above. The animals were transferred in groups of varying number and sampled as groups at varying time periods up to 40 hours after transfer. The reciprocal transfer was not investigated.

Distribution and Quantification of Sodium, Potassium, and Water

Serum sodium and potassium levels were determined by flame-photometry (Baird-Atomic, Model KY-3). After dilution of 10 ul of serum in 20 ml of lithium chloride carrier solution (250 mg/l), the samples were compared to standard NaCl and KCl solutions. The sodium and potassium contents of bile were also examined. Bile was collected with capillary pipettes from the exposed gall bladder. Flame-photometry was also used to determine total body sodium and potassium content after liquefaction of the entire body of the animal in the minimal amount of concentrated nitric acid. In addition, a few determinations on the axial muscle tissue were done in the same manner. Total body water was determined by weighing before and after drying in an oven at 105 C for 12 hours.

Apparent Sodium Space

The method of Lahlou and Sawyer (1969) and Mayer and Nibelle (1969) was used to determine apparent sodium space. Each fish received a 10 ul intraperitoneal injection of a ^{22}NaCl (Nuclear Science and Engineering Corporation) solution with an activity of 4.7 uCi/ml. One hour in a 20 ml bath was allowed for distribution of the labelled sodium. The animals were then removed from the bath, rinsed briefly in deionized water and blotted dry on tissue paper. A blood

sample was taken, and the radioactivity of 10 ul of serum, brought to a constant volume of 5 ml, was determined with a sodium iodide (Tl) crystal well scintillator (Nuclear-Chicago). The remaining serum and red blood cells in the collection pipette were then placed in a counting tube with the entire body of the fish and counted. This method of counting the non-homogenized body of the fish was shown by Potts and Evans (1967) to give the same result as homogenization and dispersion in a constant volume. Apparent sodium space was calculated using a formula similar to that given by Lahlou and Sawyer (1969):

$$V_{int} = \frac{R_{f_t}}{R_{int_t}} \qquad (1)$$

where V_{int} = the volume of distribution of sodium; R_{f_t} = the total relative activity of the fish at time t; and R_{int_t} = the relative activity per unit volume of serum at time t.

Inulin Space

The distribution volume of carboxyl-^{14}C-inulin (International Chemical and Nuclear Corporation; Lot no. 2406-30) in fish acclimated to freshwater and saltwater was determined. The fish were retrieved by netting, anesthetized in tricaine methanesulfonate (MS-222, Sandoz)

solution sufficiently concentrated to cause a torpid state in one to two minutes, and placed ventral side up on moistened tissue paper under a dissecting microscope. Two microliters per fish of isosmotic sodium chloride solution containing Evan's blue dye and 20 uCi/ml of carboxyl-^{14}C-inulin (6.4 uCi/mg) were injected into the caudal vein. No equal volume of blood was removed. The injection apparatus consisted of an eight inch long PE 60 tubing (Intramedic) connected on one end to a 50-ul microsyringe (Hamilton) and on the other end to a glass hypodermic needle. The glass hypodermic needle was made of capillary tubing (0.73 to 0.75 mm, id.) which had been heated and drawn to a fine tip measuring approximately 100 microns in diameter. The shank of the needle was twice as long as the basal portion which attached to the PE 60 tubing. The PE 60 tubing served two functions: (1) it allowed maneuverability of the hypodermic needle under the microscope unencumbered by the long microsyringe; and (2) it prevented radioactive contamination of the microsyringe. The hypodermic needle was inserted into the caudal vein in the area of the midline of the caudal peduncle region on the ventral surface of the fish at an angle of approximately 45^{o} to the vertebral column. Injection volumes were always measured from the same place

on the microsyringe. After injection, the fish were placed into a bath of exactly 20 ml of the medium to which they had been acclimated. The optimum time allowed for distribution of inulin in the fish had been established by preliminary experiments to be 15 to 20 minutes. Time periods shorter than this showed an elevated inulin space. Inulin space was the same after one hour as it was after 15 minutes. Since the radioactivity of the serum declined rapidly, the shortest time interval allowing maximum distribution was the most desirable. After distribution of the inulin, each fish was removed from its bath, and a blood sample taken. Samples of 100 ul of the distribution bath were also taken. Adsorption of inulin onto the sides of the beakers containing the 20 ml bath was shown to be negligible. After weighing the fish, dissection at the site of injection was performed. If any concentration of Evan's blue dye was found in the muscle tissue surrounding this site, the determinations for that fish were discarded. The serum and bath samples were placed on planchettes, wetted with 95% ethyl alcohol and dried with a planchette spinner equipped with an infrared lamp. Standards were prepared by placing 10 ul of non-radioactive fish serum and 2 ul of the inulin-^{14}C injectate on a planchette, mixing, and wetting with 95% ethyl alcohol. Thus,

no correction for self-absorption was made. The relative activity of the samples was determined with a thin window gas flow detector (Nuclear-Chicago). The radioactivity per unit volume of serum (I^*_{int}), the amount of inulin-^{14}C lost to the bath (I^*_{ext}), the initial activity injected (I^*_o), and the weight of the fish were used to calculate the distribution volume of inulin inside the animal. This was assumed to be an estimation of total extracellular space (TECS).

$$TECS = \frac{I^*_o - I^*_{ext}}{I^*_{int}} \qquad (2)$$

Thorson (1958, 1961) made several observations on the use of inulin to measure extracellular space which led him to believe that inulin did not penetrate minor fluid compartments in the large fishes he examined. The pericardial and coelomic fluids of the pupfish could not be collected in sufficient volume to monitor; however, injections of inulin-^{14}C into the coelomic fluid showed that inulin rapidly entered the blood stream. This technique could not be used to determine inulin space, apparently because the loss of inulin from the blood was so rapid that the coelomic fluid never reached equilibrium with the blood.

Drinking Rate

Radioactive colloidal ^{198}Au was used to determine the

the drinking rates of freshwater- and saltwater-acclimated fish. This technique was similar to that of Motais, et al (1969). To one liter of each acclimation medium, 250 uCi of colloidal ^{198}Au were added. Disposable aquaria were used for this purpose since the decay product of ^{198}Au is stable mercury. Groups of varying numbers of acclimated fish were placed in these media and allowed to drink from 1 to 2.5 hours. After this time, the animals were removed, blotted dry on tissue paper, and weighed. In some instances blood samples were removed and counted. The gut was removed and suspended in contant volume. Contamination of surgical instruments was kept to a minimum by using different instruments to cut away the body wall and to remove the gut. All surgical instruments were washed and monitored after removal of each gut. Whole body counts were not useful since the colloidal gold apparently was adsorbed onto the surface of the fish.

Thalium activated sodium iodide well scintillation (Nuclear-Chicago) was used to determine the radioactivity of the gut and its contents. During the same counting period a 100 ul sample of the drinking solution, which had been removed at the same time the fish were removed, was counted. Thus, no correction for radioactive decay was necessary.

Drinking rate was determined by comparison of the radioactivity of the gut with the radioactivity of the drinking solution. Filtration of the drinking solution through Whatman #40 filter paper in a Büchner funnel just prior to each experiment was necessary to remove fine particulate matter. Apparently the fasted fish were capable of extracting from the water and ingesting fine particulate matter to which colloidal gold had adhered. When the drinking solution was not filtered, extremely high "drinking rates" resulted. Blood samples removed from the fish used in this experiment showed no significant amount of radioactivity in the blood. Because the gut of this species is only about 1.5 times the total body length, loss of colloidal gold via the anus was evaluated by examining the distribution of radioactivity within the guts of several fish. Each gut was divided into thirds, designated the esophageal, middle, and anal segments. The percentage of total radioactivity in each segment was determined. About half of the fish were allowed to drink one hour while the other half drank for 2.5 hours. Only saltwater-acclimated fish were used in this part of the study because previous work had shown the greatest drinking rate occurred in saltwater (Lotan, 1969; Potts and Evans, 1967; Maetz and Skadhauge, 1968). If anal loss did not occur in saltwater, loss

in freshwater would be doubtful.

Steady State Sodium Kinetics

The amount and rate of sodium efflux from pupfish in freshwater and saltwater were examined with the use of $^{22}NaCl$ (Nuclear Science and Engineering Corporation). Saltwater-acclimated animals were loaded with ^{22}Na by placing each fish in an aerated 25 ml bath containing 11.3 uCi of activity. After an equilibration period of 12-14 hours, the fish were removed, rinsed for 3-5 minutes in non-radioactive medium and placed in a 20 ml efflux bath. Freshwater-acclimated fish were loaded with ^{22}Na by intraperitoneal injection of 10 ul of saline solution containing 5.65 uCi/ml radioactive sodium. After an equilibration time of 2-4 hours, the fish were removed, rinsed as above, and placed in a 20 ml efflux bath. Aliquots of 5 ml were removed from the bathing solutions at 30 minute time intervals, counted in a solid well scintillation system (Nuclear-Chicago), and returned to the efflux baths. Mixing of the bathing solutions was assured by constant aeration. After termination of each experiment a whole body count of the living fish was taken. Due to the long half-life of ^{22}Na, 2.58 years, no correction for decay was necessary.

An equation derived from that of Solomon (1960) for a two compartment, closed system was used to determine the sodium turnover rate. This equation treats the fish as one compartment with regard to sodium. Although Motais, et al (1966) and Potts, et al (1967) showed that sodium kinetics in Platichthys and Tilapia, respectively, were appropriately described by a two compartment fish, their experiments extended for much longer time periods than those reported here. The time limitation was imposed by evaporation of the efflux bath and random counting error. These factors produced insufficent accuracy in sampling and counting as the radioactivity of the bath approached its maximum value (equilibrium). Therefore, although the pupfish may in fact have two compartments, with regard to sodium, only one, the so-called fast compartment, was seen.

Since the system was closed, the total amount of radioactivity in the system (R_o) did not change; therefore,

$$R_o = R_f + R_b \qquad (3)$$

where R_f = the radioactivity of the fish and R_b = the radioactivity of the bath. Because the fish was in a medium to which it was acclimated, the system was in the steady state, and therefore, the flux of sodium out of the fish equalled the flux into the fish. It followed that

$$\frac{-dR_f}{dt} = \frac{dR_b}{dt} = k_{12}R_f - k_{21}R_b \qquad (4)$$

where k_{12} and k_{21} represent the rate constants for sodium movement out of and into the fish, respectively. Rearrangement and integration of equation (4) yields

$$R_b = R_{eq}[1-e^{-k_{12}(1 + \frac{S_1}{S_2})t}] \qquad (5)$$

where R_{eq} = the radioactivity of the bath at theoretical equilibrium, S_1 = the total sodium content (labelled plus unlabelled) of the fish and S_2 = the total sodium content of the surrounding bath. In the saltwater medium S_2 was much larger than S_1; thus, $(1 + \frac{S_1}{S_2})$ went to unity.

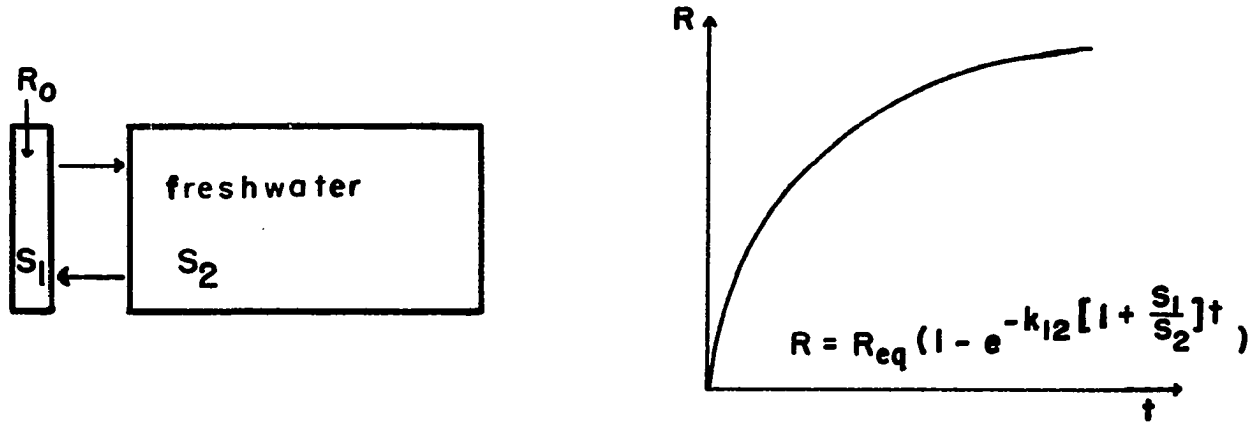
Equation (5) described the line for the appearance of ^{22}Na in the bathing solution versus time (Figure 1). The rate constant, k_{12}, could be found graphically by a semi-logarithmic plot of the $(R_{eq} - R_b)$ values versus time. When this was done k_{12} or $k_{12}(1 + \frac{S_1}{S_2})$ was the slope of the resulting straight line (Figure 2).

Lahlou and Sawyer (1969) showed that the exchangeable sodium pool (Na_p) in Opsanus was more accurately represented by

$$Na_p = (V_{int})(Na_i) \qquad (6)$$

where V_{int} = Na space and Na_i = serum sodium concentration,

Figure 1. Schematic representation of the two compartment closed system which describes the efflux of sodium from C. rubrofluviatilis in freshwater and saltwater (Solomon, 1960). The relative size of the rectangles indicates the amount of sodium in each compartment (S_1, S_2). The fish, compartment one (small rectangle), was loaded with initial activity, R_o. The radioactivity of the fish, R_f, declined with time as ^{22}Na moved into compartment two, the bath (large rectangle). The time course for the increasing activity of the bath, R_b, is shown in the accompanying graphs, with the equations for those lines. Motais (1967) described a similar system for Platichthys flesus.


R_o
freshwater
S_1
S_2
R
$R = R_{eq}(1 - e^{-k_{12}[1 + \frac{S_1}{S_2}]t})$
t

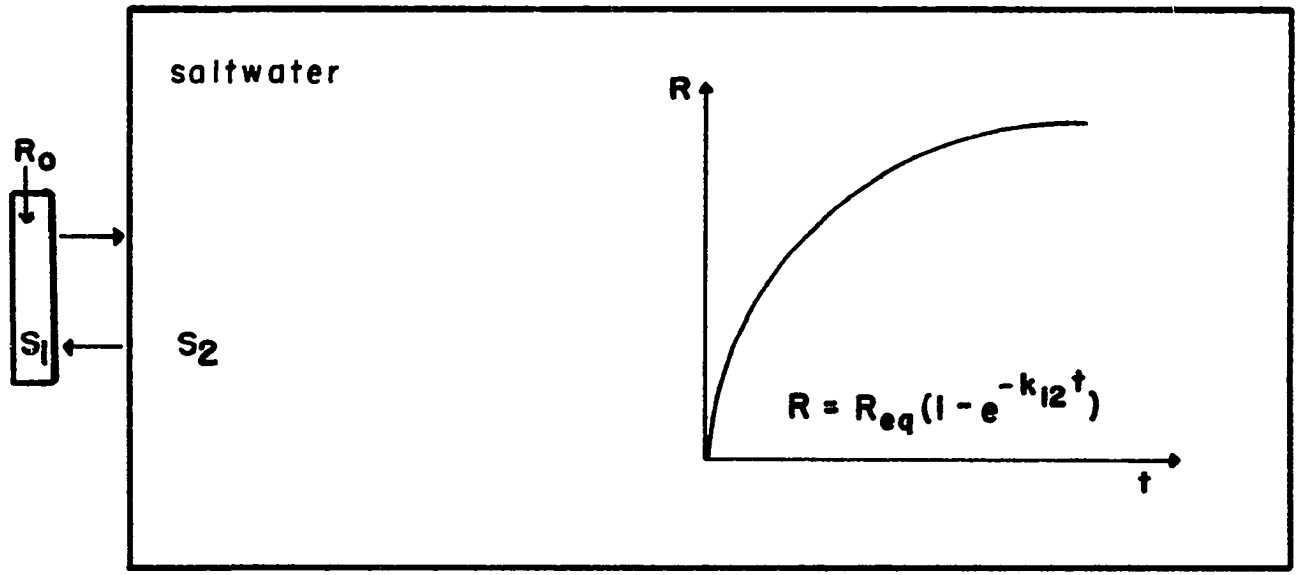
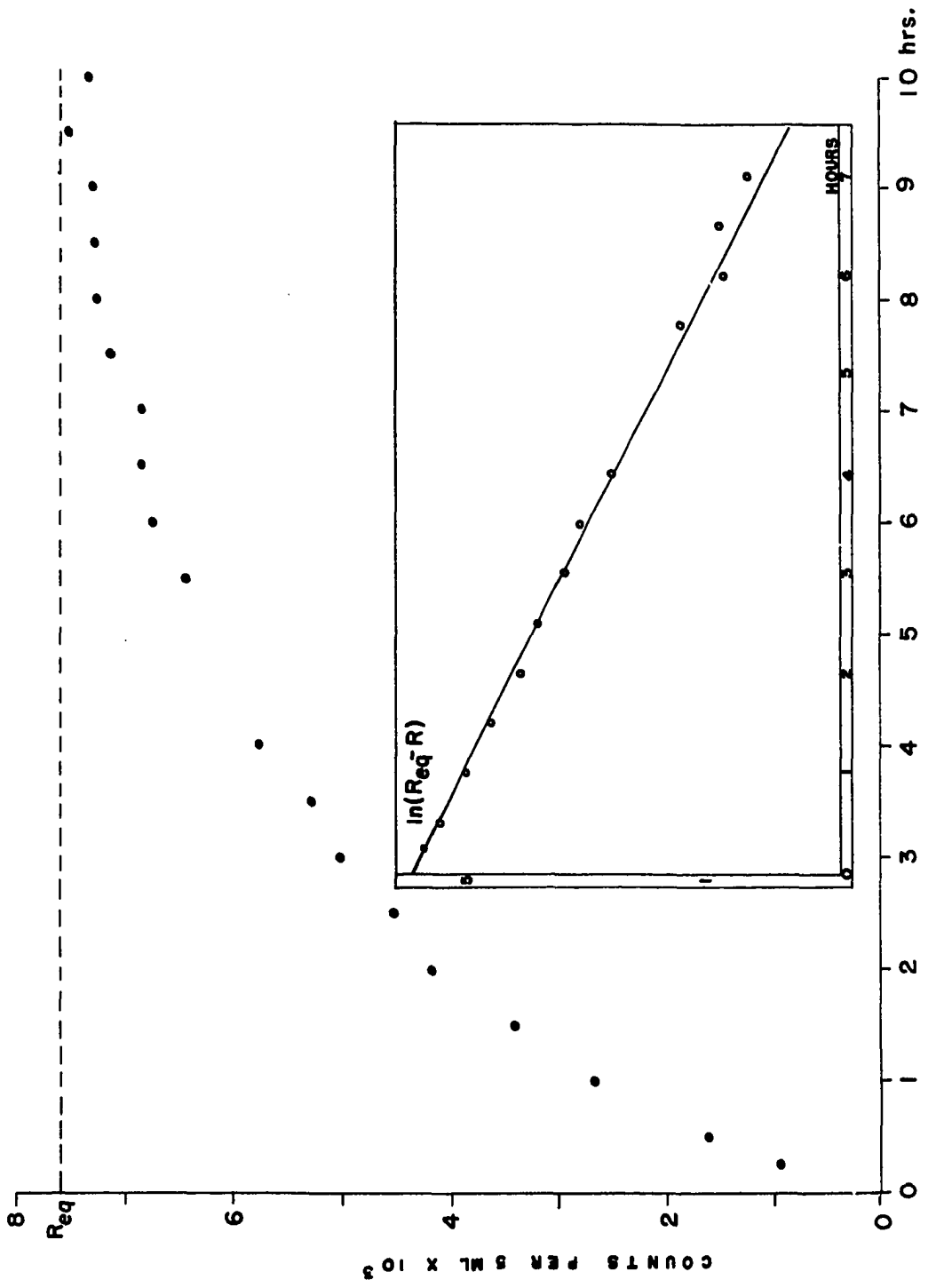

saltwater
R_o
S_1
S_2
R
$R = R_{eq}(1 - e^{-k_{12}t})$
t

Figure 2. Actual data for the appearance of ^{22}Na in an efflux bath surrounding C. rubrofluviatilis. R_{eq} is never reached but can be estimated as shown. The inset shows the arrangement of the data into the form of a straight line, the slope of which is the rate constant for the turnover of sodium.


COUNTS PER 5 ML X 10^3
R_{eq}
0
2
4
6
8
0
1
2
3
4
5
6
7
8
9
10 hrs.
$\ln(R_{eq}-R)$
HOURS

than the total body sodium content used by Potts and Evans (1967); therefore, the total sodium efflux (ϕ_{12}) was calculated as follows:

$$\phi_{12} = k_{12}(Na_p) \qquad (7)$$

Non-steady State Sodium Kinetics

Changes in the rate of sodium efflux were examined upon rapid transfer of pupfish from saltwater medium to: (1) freshwater medium, (2) a sucrose solution which was isosmotic to the saltwater solution (16 mEq/l Na; 1100 m-osmoles), (3) a medium made hyperosmotic to the saltwater solution with NaCl (706 mEq/l Na; 1584 m-osmoles), and (4) freshwater containing 100 mM calcium chloride. All fish used in these experiments were saltwater-acclimated. The fish were loaded with ^{22}Na in the manner described in the previous section. After first examining the efflux of sodium in the acclimation medium (control), the fish were rapidly transferred to one of the experimental media and then transferred back to a fresh control bath (Figure 4). The volume of bath in all cases was 20 ml. This technique was similar to that used by Motais (1967), Motais, et al (1966), and others. Because this system was not in a steady state with regard to sodium flux, these fluxes could not be measured. However, it was possible to

compare the slopes of the lines for the appearance of ^{22}Na in the external baths and calculate the percentage difference in sodium efflux rate in the various media (Motais, et al, 1966) (Figure 4). Due to the possibility that a delayed onset regulation might be induced in the experimental media and to the fact that the sodium radio-specific activity of the fish was continuously declining (Motais, et al, 1966), the fish were not retained in any of the three solutions for more than 30 minutes. The efflux baths were sampled at varying time periods in the same manner described for steady state determinations. Potts and Evans (1967) reported that handling F. heteroclitus did not seem to effect its sodium fluxes. However, Mayer and Nibelle (1970) have reported large changes in sodium outflux of the european eel, A. anguilla, due to handling.

Solutions containing identical amounts of sodium (360 mEq/l) and having the same osmotic pressures (1000 m-osmoles) but containing varying amounts of calcium chloride (0.0 mM, 14.7 mM, 29.4 mM, or 58.7 mM) were used to test the effects of external calcium concentration on sodium efflux. The 0.0 mM $CaCl_2$ solution was used as the control bath.

Gill Cytology in Varying Salinity

The cytology of the gill tissue was examined in fish

acclimated to freshwater and to saltwater. The gill tissue was fixed in Bouin's fluid, paraffin embedded, sectioned at seven microns, and stained with Harris' hematoxylin with eosin Y counterstain or Mallory's triple stain. Fish were also examined at 2, 4, 6, and 27 hour periods after transfer from freshwater to saltwater.

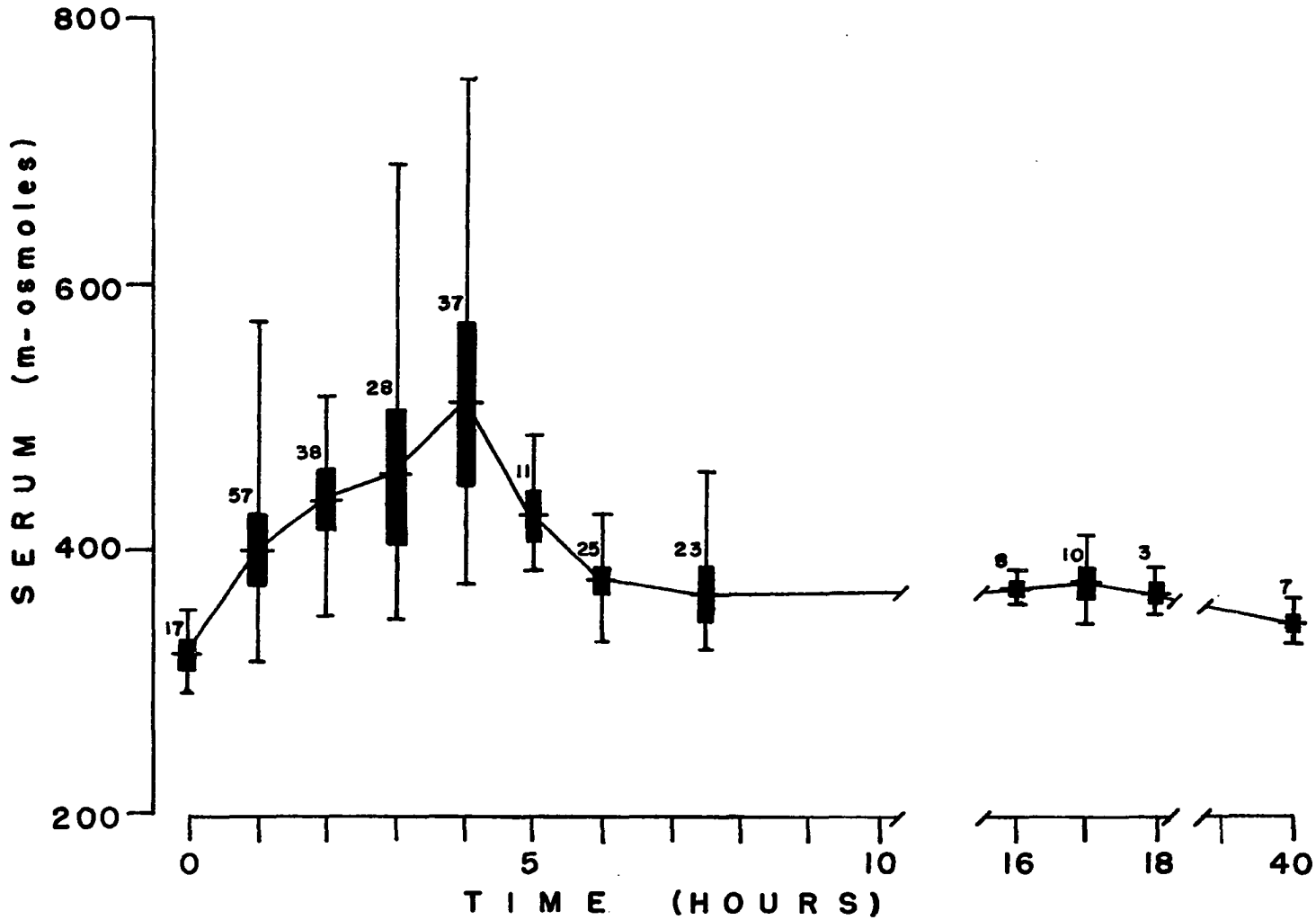
CHAPTER III

RESULTS

Serum Osmotic Pressure

Although serum osmolality varied considerably among individuals, a definite pattern of acclimation to increased salinity was discernible (Figure 3). Freshwater-acclimated fish had a serum osmotic pressure of 321 ± 5.1 (S.E.) m-osmoles (n=17). After transfer to saltwater, serum osmotic pressure rose sharply to 399 ± 7.0 (S.E.) m-osmoles (n=57), an average increase of 78 m-osmoles during the first hour. After this time, a slower increase took place (38 m-osmoles/hr). The mean peak value of 512 ± 20.0 (S.E.) m-osmoles (n=37) was reached during approximately the fourth hour. At this time values ranged from 374 to 752 m-osmoles. In the period between the fourth and sixth hours, the osmotic pressure declined rapidly (68 m-osmoles/hr). Between seven and eight hours after transfer the animals appeared to be acclimated to the saltwater situation [367 ± 7.6 (S.E.) m-osmoles (n=23)]. Mean values of serum osmotic pressure

Figure 3. Serum osmotic pressure changes in C. rubrofluviatilis after transfer at time zero from freshwater (65 m-osmoles/Kg-water) to saltwater (966 m-osmoles/Kg-water). The fish appear to be fully acclimated after eight hours. The vertical line is the range; the horizontal line is the mean; the solid bar is one standard deviation; the number of individuals tested are shown above and to the left of the solid bars.


800
600
400
200
SERUM (m-osmoles)
17
57
38
28
37
11
25
23
8
10
3
7
0
5
10
16
18
40
TIME (HOURS)

between 8 and 40 hours after transfer varied between 345 ± 4.8 (S.E.) m-osmoles (n=7) and 378 ± 7.5 (S.E.) m-osmoles (n=17)(24 hour value not shown in Figure 3) and probably represented normal fluctuations. The serum osmotic pressure in freshwater-acclimated fish averaged as much as 57 m-osmoles less than that of saltwater-acclimated fish (Table 1).

Distribution and Quantification of Sodium, Potassium, and Water

Serum sodium concentration in freshwater-acclimated fish was significantly lower than in saltwater-acclimated fish (Table 1). The difference in serum sodium content in the two groups of fish averaged 23 mEq/l. Assuming that the difference in sodium content also represents a difference in chloride content (which was not measured, but was the only other external variable), the difference in serum osmolality could be accounted for almost entirely by the change in sodium chloride content of the fish (assuming an activity coefficient of about 0.8).

Serum potassium levels in freshwater-acclimated fish were slightly significantly higher than in the saltwater group (Table 1). Both groups, however, showed the low serum potassium levels characteristic of vertebrates.

Table 1. Characteristics of external media and blood serum with regard to osmotic pressure, sodium concentration and potassium concentration in C. rubrofluviatilis acclimated to hyperosmotic and hypoosmotic media

	Hyperosmotic medium	Hypoosmotic medium	P** value
External osmotic pressure (m-osmoles per Kg water)	966 - 1130	65 - 75	--
External Na conc. (mEq/liter)	424	42	--
External K conc. (mEq/liter)	5	5	--
Serum osmotic pressure (m-osmoles per liter)	378 ± 7.5* (17)	321 ± 5.1 (17)	< 0.0001
Serum Na conc. (mEq/liter)	204 ± 3.8 (18)	181 ± 6.2 (15)	< 0.01
Serum K conc. (mEq/liter)	4.3 ± 0.5 (18)	6.5 ± 0.6 (15)	< 0.02

*Mean ± standard error (n)
**Student's t-test

The total body sodium content in both freshwater- and saltwater-acclimated fish was high (Table 2). Although the high sodium values in saltwater-acclimated fish might be explained by sodium in the gut or sodium adhering to the surface of the fish, this was not a possibility in freshwater-acclimated fish. The total body sodium of freshwater-acclimated fish was slightly significantly lower than the total body sodium of saltwater-acclimated fish (Table 2). Another possible explanation of the high total body sodium values would be a large concentrated sodium pool(s) within the fish. Because the gall bladder in this species was quite large and always distended, its sodium content was determined. The sodium concentration of bile was much higher than that of serum in both media (Table 2), but averaged 3.4% of the apparent sodium space. Muscle tissue alone also showed a high sodium content (Table 2). Of course, interstitial fluid and some blood and bone were included with the muscle, but it was doubtful that these would cause such high sodium values.

Total body and muscle potassium values were notably not significantly different in the two media (Table 2). The values of total body water in freshwater-acclimated fish were slightly larger but not significantly larger than those

Table 2. Total body sodium, potassium, and water; bile sodium concentration; and muscle sodium and potassium content in C. rubrofluviatilis acclimated to hyperosmotic and hypoosmotic media

	Hyperosmotic medium	Hypoosmotic medium	P** value
Total body Na (mEq/Kg)	115.1 ± 5.6* (10)	95.3 ± 4.9 (10)	<0.02
Total body K (mEq/Kg)	44.2 ± 1.3 (10)	45.6 ± 2.4 (10)	<0.9 >0.5
Bile Na (mEq/liter)	325.3 ± 5.8 (3)	302.4 ± 12.0 (5)	<0.3 >0.2
Muscle Na (mEq/Kg)	91.4 ± 12.1 (5)	61.2 ± 8.8 (6)	<0.001
Muscle K (mEq/Kg)	58.2 ± 7.3 (5)	47.8 ± 5.2 (6)	<0.3 >0.2
Total body water (% body weight)	77.8 ± 0.5 (10)	79.5 ± 0.9 (10)	<0.2 >0.1

*Mean ± standard error (n)
** Student's t-test

acclimated to saltwater (Table 2). This indicated that the differences in ionic content of the two groups of fish were absolute differences and not a result of dehydration.

Apparent Sodium Space; Extra- and Intracellular Space

The total extracellular space (inulin space) was the same in fish acclimated to either medium (Table 3). The values for freshwater sodium space were only slightly larger than the values obtained for freshwater extracellular space (Table 3). Upon acclimation to saltwater, sodium space greatly increased, but extracellular space remained unchanged (Table 3). Because total body water was unchanged, intracellular space also remained the same in both media (Table 3). The result appeared to be that intracellular sodium increased greatly upon acclimation of the fish to saltwater.

Drinking Rate

Drinking rate for fish in saltwater averaged about 1.4% of the body weight per hour during test periods varying between 1 and 2.5 hours (Table 4). Assuming all the sodium swallowed was absorbed, approximately 6.0% of the total exchangeable sodium pool was replaced by drinking every hour. In freshwater, significantly less of the medium was swallowed

Table 3. Sodium space, extracellular and intracellular space in C. rubrofluviatilis acclimated to hyperosmotic and hypo-osmotic media

	Hyperosmotic medium	Hypoosmotic medium	P** value
Sodium space (ml/100g)	50.9 ± 2.8* (6)	30.9 ± 2.3 (7)	<0.001
Extracellular space (inulin space, ml/100g)	25.2 ± 0.9 (9)	24.9 ± 1.4 (14)	>0.5
Intracellular space (total water minus inulin space, ca. ml/100g)	52.6	54.6	

*Mean ± standard error (n)
**Student's t-test

Table 4. Rate of drinking and calculated rate of sodium gained from drinking of C. rubrofluviatilis in hyperosmotic and hypoosmotic media. The distribution of colloidal ^{198}Au within the gut of saltwater-acclimated fish is shown

	Hyperosmotic medium	Hypoosmotic medium	P** value
Drinking rate (ml/Kg/hr)	14.4 ± 3.5* (20)	2.0 ± 0.4 (12)	< 0.02 > 0.01
Sodium gained by drinking (mEq/Kg/hr)	6.12	0.08	

Gut segment	% radioactivity per segment
Esophageal segment	70.9 ± 5.1 (9)
Middle segment	21.0 ± 4.4 (9)
Anal segment	8.1 ± 2.0 (9)

*Mean ± standard error (n)
**Student's t-test

(0.2% body weight per hour). This rate of drinking amounted to only 0.14% of the total exchangeable sodium pool per hour.

Loss of colloidal gold-198 via the anus was slight in the time period allowed for drinking. No distinguishable difference in distribution could be seen with respect to the two time periods used (1 and 2.5 hours); therefore, the data for all fish were combined. Most of the radioactivity of the gut was located in the esophageal segment (Table 4). Only a small amount was found in the anal segment.

Steady State Sodium Kinetics

The sodium turnover rate for fish in saltwater amounted to nearly one-half of the exchangeable sodium in the fish per hour (Table 5). The turnover rate was much lower in freshwater, but only slightly less than 40% of the turnover rate in saltwater (Table 5).

Non-steady State Sodium Kinetics

Upon rapid transfer of saltwater-acclimated fish into freshwater, a decrease in sodium efflux of over 50% occurred (Figure 4 and Table 6). This was an instantaneous decrease, which did not persist after transfer back to saltwater. Some decrease was seen in the second control bath but this could be explained by the decrease in radio-specific activity

Table 5. Sodium efflux rate constants and total sodium efflux in C. rubrofluviatilis acclimated to hyperosmotic and hypoosmotic media

	Hyperosmotic medium	Hypoosmotic medium	P** value
Efflux as %/hr of exchangeable Na	43.2 ± 2.6* (17)	17.2 ± 2.2 (7)	<0.001
Total Na efflux (mEq/Kg/hr)	44.9 ± 2.7 (17)	9.6 ± 0.9 (7)	<0.001

*Mean ± standard error (n)
**Student's t-test.

of the fish in control transfers (Figure 5). Since the instantaneous drop in sodium efflux in freshwater could have been explained by the decrease in either the sodium content of the freshwater or the osmotic pressure of the freshwater, the fish were also tested in a sucrose solution which was isosmotic to the saltwater solution (Figure 6 and Table 6). Sodium efflux in the sucrose solution decreased to one-third of its control value. This was a greater drop than that which took place in freshwater, probably because the sucrose solution contained only 16 mEq/l sodium.

Transfers from the saltwater acclimation solution into a hyperosmotic saline solution containing 706 mEq/l sodium and having an osmotic pressure of 1584 m-osmoles, resulted in no change in the rate of sodium efflux (Figure 7 and Table 6).

Some experiments of a preliminary nature were done testing the effect of $CaCl_2$ on the rate of sodium efflux. Transfers of fish from solutions containing no added $CaCl_2$ to solutions of 14.7, 29.4, or 58.7 mM $CaCl_2$ resulted in decreases in sodium efflux rate of 13% (n = 2), 23% (n = 1) and 19% (n = 2), respectively. Transfers from saltwater to freshwater containing 100 mM $CaCl_2$ resulted in a 65%

Figure 4. Effect of transfer to freshwater on sodium flux in C. rubrofluviatilis acclimated to saltwater (966 m-osmoles). If a_1, a_2, and a_3 are the slopes of the lines for the appearance of ^{22}Na in each bath, the relative rate (r) of sodium efflux in the experimental bath can be determined as follows:

$$r = \frac{2a_2}{a_1+a_3}$$

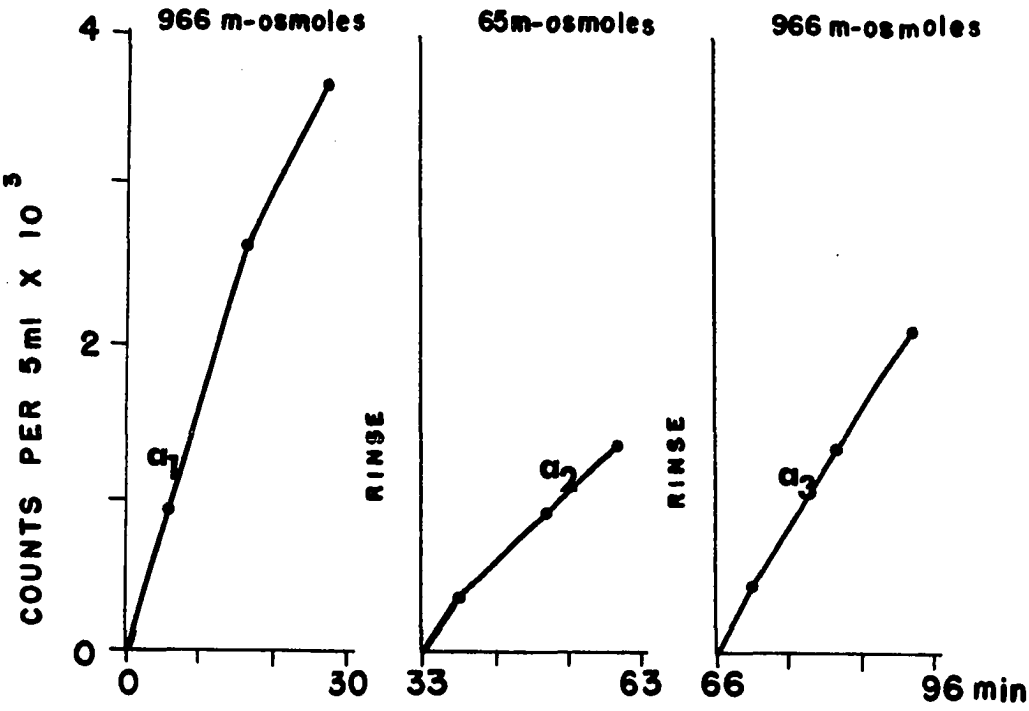

966 m-osmoles
65m-osmoles
966 m-osmoles
COUNTS PER 5ml X 10³
4
2
0
RINSE
RINSE
a1
a2
a3
0
30
33
63
66
96 min

Figure 5. Effect of transfer to the same concentration on sodium efflux rate from C. rubrofluviatilis.

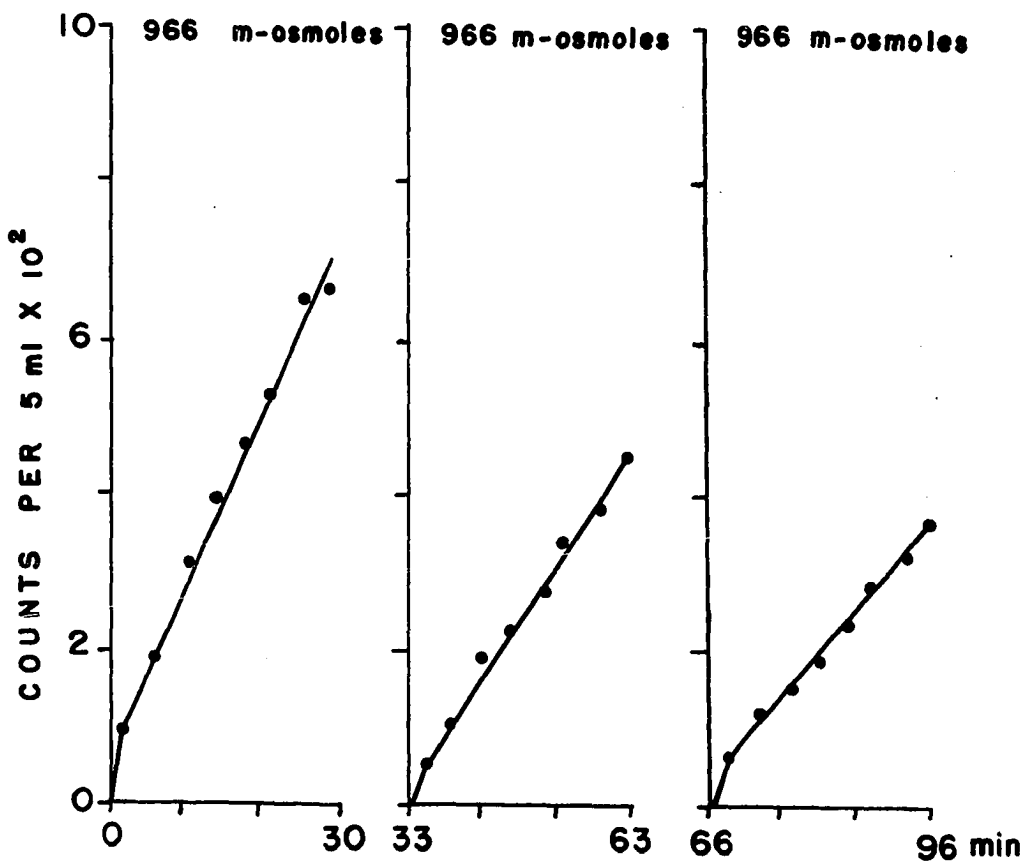

966 m-osmoles
966 m-osmoles
966 m-osmoles
COUNTS PER 5 ml X 10²
10
6
2
0
0
30
33
63
66
96 min

Figure 6. Effect of transfer from saltwater medium to isosmotic sucrose medium on sodium efflux rate from C. rubrofluviatilis.

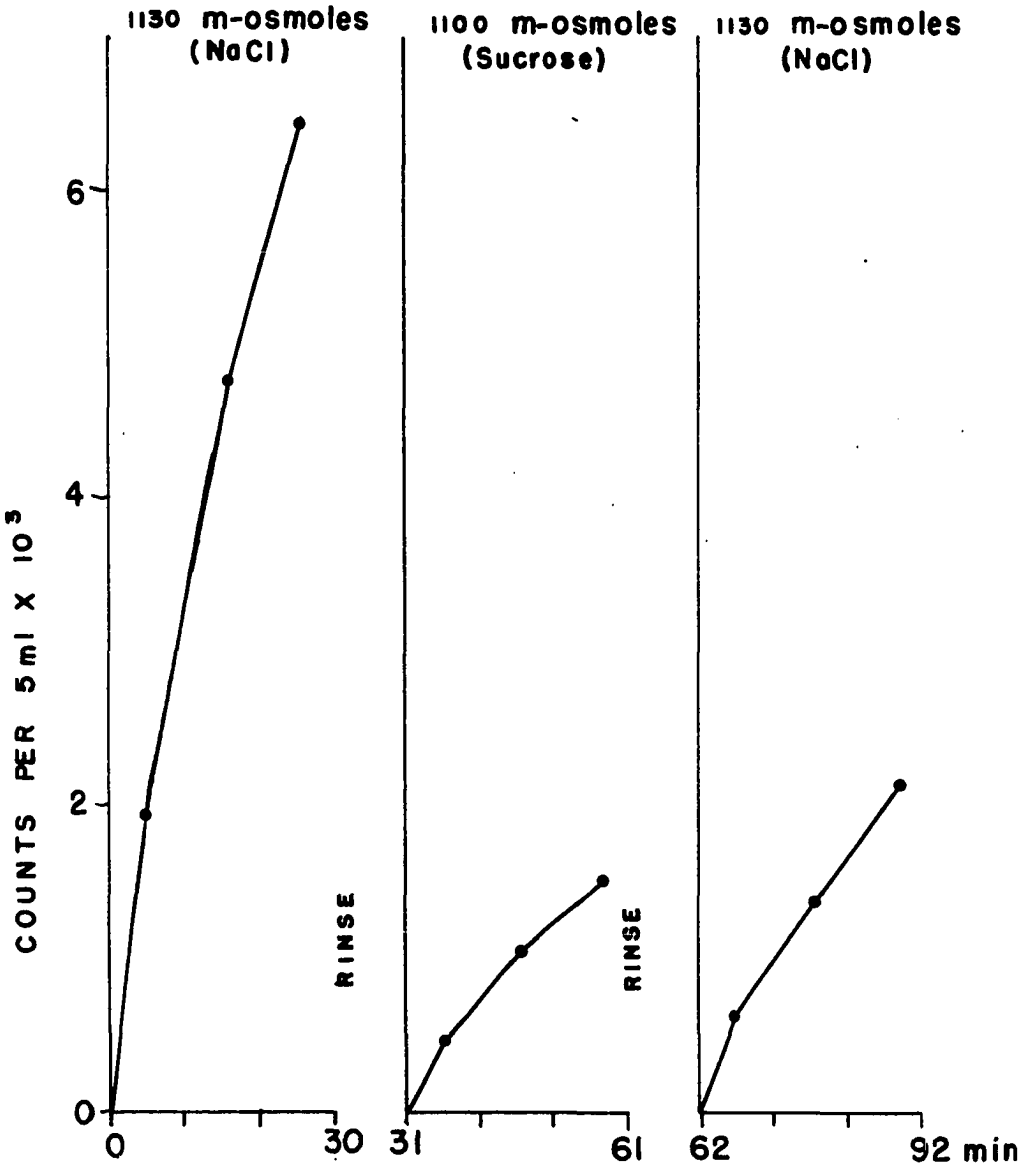

1130 m-osmoles
(NaCl)
1100 m-osmoles
(Sucrose)
1130 m-osmoles
(NaCl)
COUNTS PER 5ml X 10³
6
4
2
0
RINSE
RINSE
0
30
31
61
62
92 min.

Figure 7. Effect of transfer to a higher salinity on sodium efflux rate from C. rubrofluviatilis acclimated to water of 966 m-osmole.

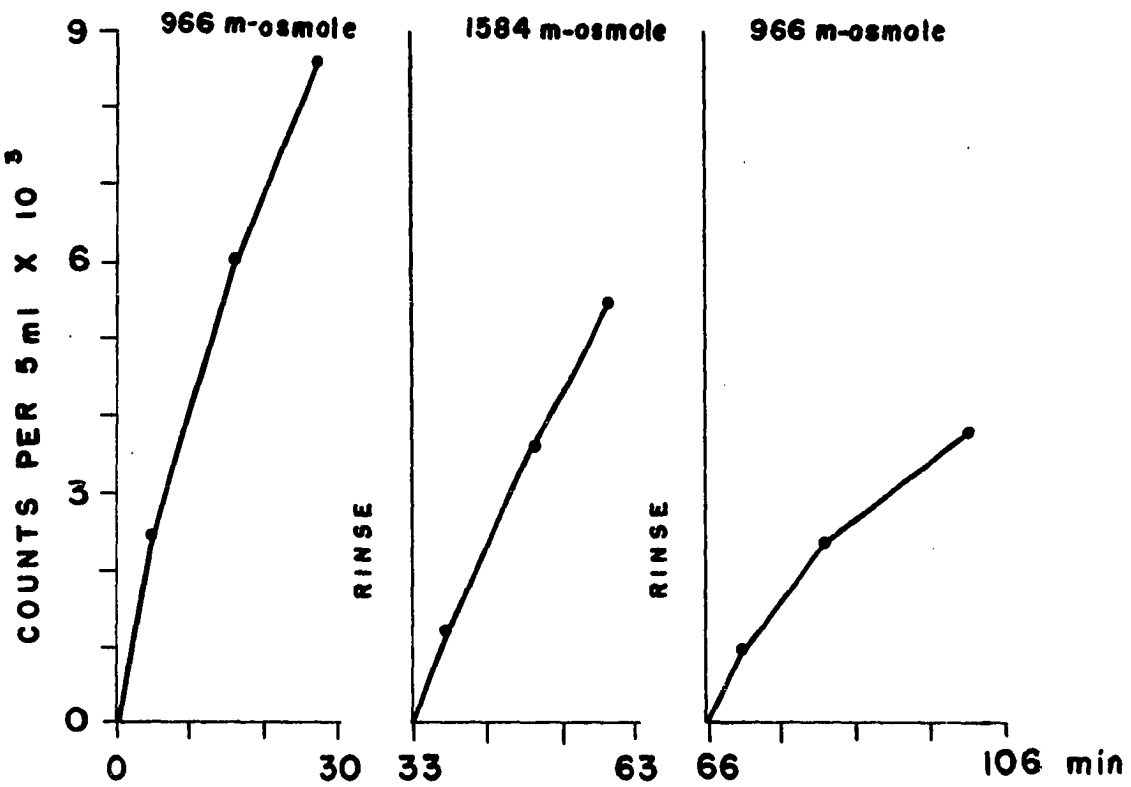

966 m-osmole
1584 m-osmole
966 m-osmole
COUNTS PER 5ml X 10³
9
6
3
0
RINSE
RINSE
0
30
33
63
66
106 min

Table 6. Changes in intensity of sodium efflux upon rapid transfer of C. rubrofluviatilis to media of different osmotic and ionic composition. The values are expressed as percent of the efflux rate of sodium in the control solution

Media transferred to	Control media	% of control value
Freshwater (42 mEq/l Na)	Saltwater (424 mEq/l Na)	48.7 ± 2.1 (12)*
Saltwater + NaCl (706 mEq/l Na)	"	95.1 ± 5.8 (7)
Sucrose solution (1048 m-osmoles/Kg-water; 16 mEq/l Na)	"	33.8 ± 2.7 (6)

*Mean ± standard error (n)

(n = 3) decrease in sodium efflux rate. Apparently short term exposure to $CaCl_2$ does have a slight effect on the sodium efflux rate. Since sodium efflux was uninhibited by high chloride concentrations in saltwater, it was not likely that the high chloride concentrations in the $CaCl_2$ solutions caused the decline in sodium efflux. For this reason these preliminary data were interpreted as a calcium effect.

Gill Cytology in Various Salinities

Three main cell types were found in the outer layer of the filament tissue. Plate-like epithelial cells covered the primary and secondary filaments. Goblet shaped cells were frequently found on the filaments, but usually not in abundance. The goblet shaped cells were assumed to be mucous cells. The third cell type which was very large and highly eosinophilic, was located primarily between the secondary lamellae (Figure 8A). These cells answered the exact description of the so-called chloride or Keys-Willmer cells (Keys and Willmer, 1932; Getman, 1950; Doyle and Gorecki, 1961; Threadgold and Houston, 1961; Vickers, 1961; Virabhadrachari, 1961; Burns and Copeland, 1950; Datta Munshi, 1964; Copeland, 1948). The cells stained violaceous with Mallory's triple stain, which was an indication of glandular tissue (Davenport, 1960).

Figure 8 (Plate). A. Transverse section of a primary filament of a saltwater-acclimated C. rubrofluviatilis. The position of the Keys-Willmer cells (k-w) is shown with respect to the afferent (a) and efferent (e) arterioles and the secondary lamellae (sl) (X430). B. Sagittal section of a primary filament showing an apical crypt (ac) shared by two Keys-Willmer cells (X930). C. Sagittal section of a primary filament of a saltwater-acclimated pupfish showing the normal arrangement of the cells between the secondary lamellae. Note the large nuclei (n) (X930). D. Sagittal section of a primary filament of a freshwater-acclimated C. rubrofluviatilis. Note the absence of apical crypts (X930).

The cells seemed to stain with equal intensity whether from fish acclimated to freshwater or saltwater. The only major difference in the cells in the two media was the presence of a large apical crypt in the end of the cell in contact with the external medium (Figure 8B, 8C). Frequently two cells shared the same apical crypt (Figure 8B). This crypt was rarely present in freshwater-acclimated fish (Figure 8D).

Circumstantial and some direct evidence has accumulated which relates these cells to osmoregulation, specifically chloride regulation (Conte and Morita, 1968; Philpott, 1966; Conte, 1965). These cells were very abundant in the pupfish whether in freshwater or saltwater. No evidence was obtained which directly related them to osmoregulation in the pupfish; however, the development of the apical crypt was found to be almost coincidental with the onset of osmotic regulation after transfer from freshwater to saltwater. The above conclusion was reached by a subjective analysis of the frequency of apical crypts in gill sections taken at various times after freshwater to saltwater transfer. Objective analysis was impossible due to the nature of the material. There appeared to be a progressive increase in the number of crypts from two hours to six hours. Very little difference could

be noted between 6 hours and 27 hours. It must also be pointed out that apical crypts were infrequently found in freshwater-acclimated fish. Transfers of saltwater-acclimated fish back to freshwater showed that the apical crypts remained frequent for up to eight days after transfer.

CHAPTER IV

DISCUSSION

The serum osmotic pressures of pupfish in freshwater and saltwater were within the normal ranges expected for euryhaline teleosts in similar media (Parry, 1961; Hickman, 1959; Yamashita, 1970a,b; and Lange and Fugelli, 1965). The serum osmotic pressures for the pupfish in saltwater stabilized at values approximately 18% greater than the values for fish in freshwater. Pleuronectes and Gasterosteus (Lange and Fugelli, 1965) showed comparable increases in serum osmolality in saltwater of 20% and 17%, respectively, while Salmo salar showed a change of only 5% (Parry, 1961).

The initial serum osmo-concentration after abrupt transfer of pupfish from freshwater to saltwater probably resulted from both a loss of water and a gain of sodium and chloride. During acclimation the fish apparently increased water uptake, partially by drinking, and increased active elimination of sodium chloride.

The rapidity of acclimation to increased salinity was notable. A decrease in the rate at which the serum osmolality increased could be seen after one hour. The serum osmotic pressure began to decline during the fourth hour. After only eight hours the serum osmotic pressure had stabilized. This was a much shorter time than that required by S. gairdnerii (80-170 hours, Houston, 1959); or the eel, A. anquilla (50 hours, Keys, 1933), under similar conditions. The abrupt transfer study indicated that C. rubrofluviatilis was a very efficient and tolerant osmoregulator.

Serum sodium levels in the pupfish were comparable to values obtained for other teleosts (Phillips and Brockway, 1958; Parry, 1961; Fromm, 1963; Chan, et al, 1967; Butler, 1966). The increased serum osmotic pressure in saltwater-acclimated fish was probably in large part caused by an increase in serum sodium and chloride content. The comparatively small difference in serum sodium levels in freshwater and saltwater indicated an efficient sodium regulatory mechanism.

The serum potassium levels were slightly larger in freshwater than in saltwater. This same situation was shown in F. kansae (Stanley and Fleming, 1964) and O. kisutch (Miles and Smith, 1968), but the opposite was true of P.

flesus (Lahlou, 1967) and A. anguilla (Chan, et al, 1967). The decreased serum potassium level in saltwater-acclimated fish may be due to the movement of potassium into the cells since muscle potassium content increased in saltwater but total body potassium content was the same in both media.

The total sodium content in fishes of slightly larger or similar size to the pupfish and in similar media (Foster, 1969; Potts and Evans, 1967; and Lotan, 1969) averaged much below the values obtained for pupfish. From results of bile analysis, it was demonstrated that the high sodium values for the pupfish may in part result from concentrated sodium pools within the fish. However, axial muscle tissue also showed relatively high sodium levels. Total body potassium was low compared to total body sodium content. Since the diet of the fish contained ample potassium (100 mEq/Kg dry weight), the low body potassium levels were not due to lack of availability of this ion. These data indicated that C. rubrofluviatilis normally has a high sodium to potassium ratio.

This work partially answers the question raised by Mayer and Nibelle (1969) as to whether the increase in sodium space seen in saltwater-acclimated fishes was due to an increase in extracellular space or an increase in cellular sodium. In the pupfish there was a substantial increase in

cellular sodium after saltwater acclimation. This appeared to have no debilitating effect on the animals. Because of the osmotic gradient, one of the initial effects of saltwater on freshwater acclimated fishes is to dehydrate them (Gordon, et al, 1965). If the cellular membranes are more permeable to water than to salts, the cells must lose water to remain isosmotic to the extracellular fluid. Movement of sodium ions into the cells would in effect conserve the cell water and stabilize the cell volume. As Mayer and Nibelle (1969) stated, this process would dilute the increased plasma sodium in saltwater and minimize large osmotic changes. Although cellular sodium increased in saltwater, the ratio of total cation concentrations (K and Na only) inside and outside the cells was almost the same in saltwater as in freshwater-acclimated fish (0.509 and 0.502, respectively).

The work of Lahlou, Henderson, and Sawyer (1969) on the stenohaline freshwater goldfish, Carassius auratus, showed that in a hyperosmotic medium extracellular space was almost doubled compared to the values obtained for the same species in freshwater. The small increase in sodium space they recorded was a result of the increased extracellular space, not a consequence of the movement of sodium into the cells. Mayer and Nibelle (1969) showed that the eel, A. anquilla,

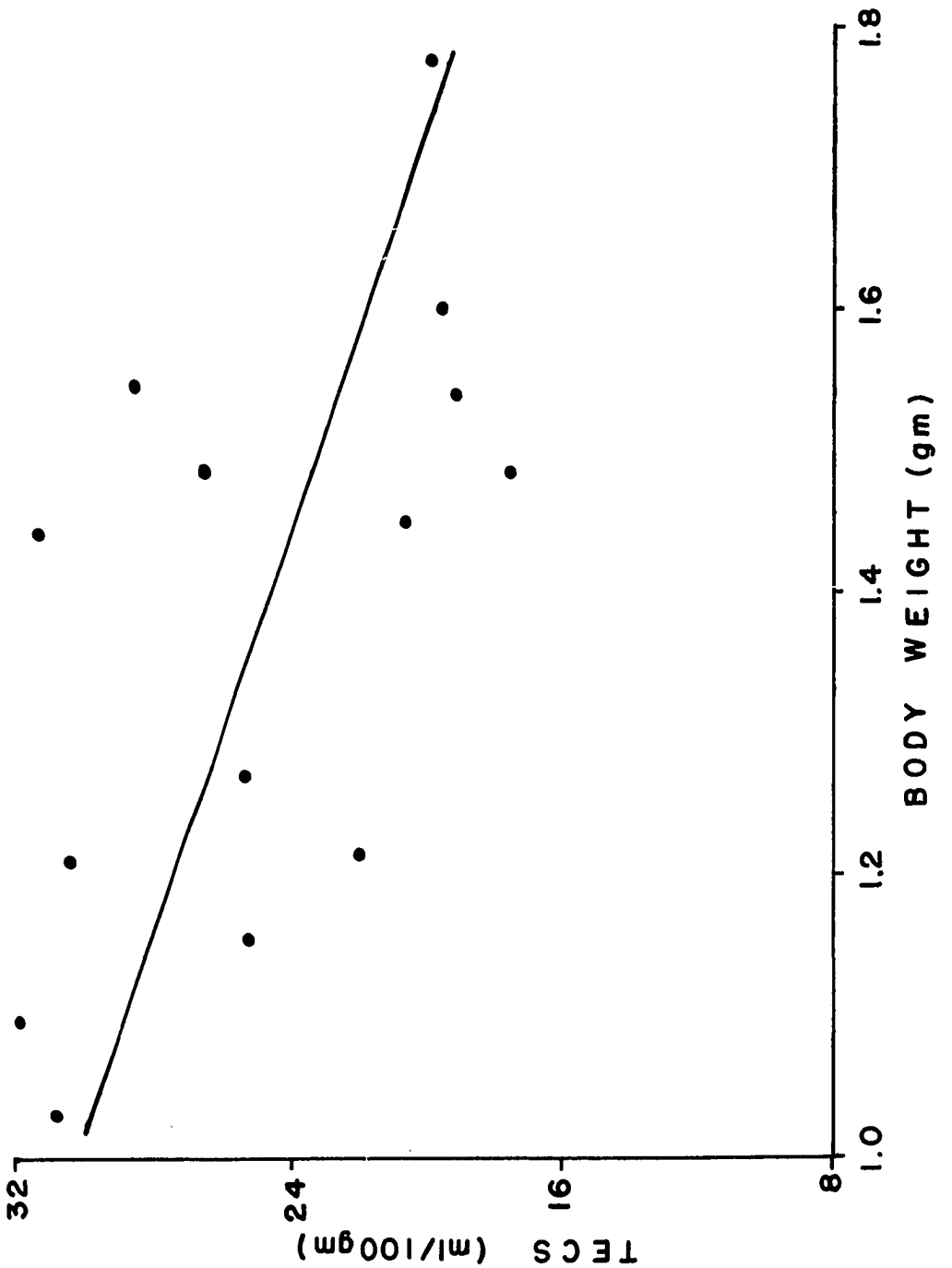
underwent a significant increase in sodium space in salt-water (22.6 to 29.1 ml/100g). Chan, et al, (1967) showed that muscle extracellular space increased from 7.7 ml/100g to 16.15 ml/100g (recalculated approximations) in the yellow eel after freshwater to sea water transfer, and from 10.25 ml/100g to 12.46 ml/100g in the silver eel under similar conditions. Mayer and Nibelle (1969) did not specify if their eels were yellow or silver. If they were yellow eels, the increased sodium space in sea water could probably be accounted for by an increase in extracellular space. This might not be true of the silver eel, for this form of A. anguilla is the better osmoregulator. Evans (1967) showed a relatively constant extracellular space in the blenny, Xiphister atropurpureus, in 10 to 100% sea water. Sodium space was not measured by Evans (1967) but serum sodium did increase about 20% in his animals after transferral from 10 to 100% sea water.

Extracellular space recorded for C. rubrofluviatilis was relatively large compared to the values obtained for most fishes tested. Xiphister had an extracellular space only half as large as the pupfish (Evans, 1967). Gordon, et al (1965) reported an extracellular space for the mud-skipper, Periopthalmus sobrinus, which was similar to that

obtained for the pupfish. To my knowledge, extracellular space has never been determined on fishes as small as C. rubrofluviatilis (0.5 to 2.0g). Thorson (1961) recorded very little difference in the extracellular space of marine and freshwater teleosteans (15 and 14% body weight, respectively). He used no teleosts which weighed less than one thousand grams. Thorson (1961) stated that some series of measurements in his work indicated a proportionately larger blood volume in smaller fish. Martin (1950) had shown this to be true with the dogfish shark, Squalus sucklii. Recalculation of the values for extracellular space reported for the hagfish, Myxine (Cholette, Gagnon, and Germain, 1970) showed that inulin space per unit body weight tended to decrease as body weight increased. An indication of this same relationship was seen in C. rubrofluviatilis (Figure 9). Due to the narrow weight range, the relationship of body weight to TECS showed only a slightly significant negative correlation [$r = -0.5936$; $P(r = 0) < 0.05$]. Not enough data exist at this time to make a general statement on this point.

Drinking rates for fishes in sea water normally range from near zero to 2% body weight per hour (Foster, 1969; Lotan, 1969; Potts and Evans, 1967; Dall and Milward, 1969; Evans, 1967; Potts, et al, 1967; Maetz and Skadhauge, 1968;

Figure 9. The relationship of total extracellular space (TECS) to body weight in C. rubrofluviatilis. The regression equation for the line shown was $Y = 44.2 - 14.0X$, and the correlation coefficient was -0.5936 ($P < 0.05$).


32
24
16
8
TECS (ml/100gm)
1.0
1.2
1.4
1.6
1.8
BODY WEIGHT (gm)

Evans, 1969a; and Motais, et al, 1969). The hourly drinking rate of pupfish in a medium similar to sea water averaged about 1.4% body weight, which puts them in the upper range of values reported for other species. The drinking rate of freshwater-acclimated pupfish was large compared to that of most other fishes; however, higher rates were reported for F. heteroclitus (Potts and Evans, 1967), Pelates, Periopthalmus (Dall and Milward, 1969), and A. dispar (Lotan, 1969). Dall and Milward (1969) have stated that regulation of water uptake was controlled by the gut wall, not the rate of drinking. Smith (1930) calculated that about three-quarters of the water swallowed was absorbed.

The increased drinking rate and, thus, the increased amount of sodium taken into the gut (6.12 mEq/Kg/hr) accounted for about 14% of the total sodium turnover in saltwater (44.9 ± 2.7 mEq/Kg/hr). If it is assumed that in steady state conditions the sodium influx and efflux are equal, the remaining 86% (34.1 mEq/Kg/hr) must enter through the gill epithelium since the body wall seems impermeable (Fromm, 1968). Although the drinking rate of A. dispar (Lotan, 1969) was slightly higher than that of the pupfish, the relative percentages of sodium influx via the gut and gills were almost identical. The reason for this was, of course, that

Aphanius had a larger total sodium flux (68.5 uM/g/hr). The rate constant for sodium turnover in pupfish in saltwater was similar to those obtained for Pelates, Periopthalmus, (Dall and Milward, 1969), P. flesus (Motais, et al, 1966), and F. heteroclitus (Potts and Evans, 1967). The rate was much lower than that obtained for Aphanius (Lotan, 1969) and much larger than those reported for Pholis gunnelis (Evans, 1969a), Xiphister (Evans, 1967), Cottus scorpius (Foster, 1969) or Opsanus tau (Lahlou and Sawyer, 1969).

The sodium turnover rate for pupfish acclimated to freshwater was very high (17.2 ± 2.2 %/hr). This was partially due to the large sodium content of the freshwater (42 mEq/l, equivalent to 10% sea water). However, this turnover rate was comparable to the turnover rate of F. heteroclitus in 40% sea water (Potts and Evans, 1967) and P. flesus in 50% sea water (Motais, et al, 1966). One might speculate that the pupfish in comparable salinities of 40 to 50% sea water would have a higher sodium turnover rate than the above two fishes. The more closely related A. dispar had very high sodium turnover rates in sea water and freshwater compared to other fishes (Lotan, 1969).

Only about 0.8% of the total sodium flux in freshwater acclimated pupfish was accounted for by drinking.

The remaining sodium was probably gained via the gills.

The following factors indicated that C. rubrofluviatilis possesses the same type of branchial sodium regulation as that seen in the euryhaline flounder, P. flesus (Motais, 1967; Motais et al, 1966) and the brine shrimp, Artemia salina (Thuet, Motais, and Maetz, 1968): (1) Upon abrupt transfer of pupfish from saltwater to freshwater the rate of sodium efflux instantaneously decreased; (2) the rate of sodium efflux decreased as the ambient sodium content decreased; (3) sodium efflux rate was not affected by external osmotic pressure; (4) variations in external chloride concentration apparently had no effect on sodium efflux rate.

The immediate decline in sodium efflux rate, when external salinity declined, was thought by Motais, et al (1966), to be due to the cessation of a sodium-sodium exchange diffusion mechanism. The uphill transport of ions was thought to be coupled with the downhill transport in a one for one exchange, which resulted in no net flux. Subsequently, Maetz (1969) has reported that the sodium pump in the saltwater acclimated teleost gill appears to be a potassium-sodium exchange pump. The hypothetical carrier exchanges internal sodium, for which it has a low affinity, for external potassium, for which it has a high affinity. The high sodium

turnover rates in sea water-acclimated fishes would appear to be due to a competition of the abundant ambient sodium with the sparse ambient potassium. Maetz (1969) has shown that potassium does have a direct effect on sodium kinetics. This theory, however, does not explain why there was no buildup of potassium within the animal.

Smith (1969) has shown that exchange diffusion of sodium does not occur in the brine shrimp. Furthermore, he has produced evidence indicating that sodium ion movement across the gill of A. salina was passive and independent. The immediate decline in sodium efflux after exposure to media of decreased sodium content was a result of changes in the electrical potential difference and diffusional permeability of the gill epithelium. Smith (1969) did show that chloride ions undergo exchange diffusion. W.T.W. Potts (personal communication) has stated that the sudden decrease in sodium efflux in sea water acclimated fish transferred abruptly to freshwater may be explained by a cessation of the sodium pump and a change in the electrical potential difference across the gill. The results of Smith (1969) have cast doubt on the explanation of sodium kinetics in fish proposed by Motais, et al (1966) and by Maetz (1969). Some indirect evidence obtained in the present report tended to support

the idea of a carrier mediated transference of sodium advanced by Maetz (1969) and Maetz, et al (1969). When C. rubrofluviatilis was transferred from saltwater to a greater salinity to which it was not acclimated, no increase in sodium efflux rate occurred. These data indicated that a limiting factor of some kind must be altered before the sodium efflux rate can increase.

In the present study, it was seen that Ca^{++} had the effect of slightly reducing the rate of movement of sodium. This effect of calcium has not been previously demonstrated in any fish. In fact, Motais, et al (1966) assumed Ca^{++} had no effect on ion fluxes in the flounder. Breder (1933) has shown that certain marine fishes could survive transfer to freshwater saturated with calcium carbonate or calcium sulfate, but they could not survive transfer to untreated freshwater. Pickford, et al (1966) have shown that addition of $CaCl_2$ to the external medium promoted freshwater survival of hypophysectomized F. kansae but not hypophysectomized F. heteroclitus. In F. kansae survival was associated with maintenance of normal serum sodium levels. Evans (1969b) and Motais, et al (1969) showed that freshwater fishes were more permeable to water than marine fishes. W.T.W. Potts (personal communication) has linked the increased Ca^{++} content of the marine environment to the reduction in water permeability.

The large eosinophilic cells seen in the gills of C. rubrofluviatilis would seem to be identical to those of C. variegatus examined by Karnacky (see p. 270, Conte, 1969). Hiltological studies by Conte (1965) and Philpott (1966) and the measurements of electrical potential difference across the gill epithelium by House (1963) and Motais and Maetz (1965), have produced evidence that these cells were involved in osmoregulation, specifically anion regulation. The significance of the apical crypt is not known. The saltwater induced formation of the crypt implies an osmoregulatory function. The crypt would obviously serve to increase the amount of surface area in contact with the external medium.

In conclusion, C. rubrofluviatilis appears to fall into neither the category of "osmoconformer" nor "osmoregulator" (Motais, 1967). The intracellular sodium content in saltwater-acclimated fish was greatly increased, but the total body sodium, potassium, and water changed relatively little. Within the range of salinities tested this fish was capable of extremely fast and efficient regulation of its serum osmotic pressure. Thus, this fish demonstrated characteristics of both an osmoconformer and an osmoregulator.

The rather limited geographical range of C. rubrofluviatilis was thought by Echelle (1970) to be due to its

adaptation to a broad ecological niche resulting in a great deal of heterospecific competition in waters of low salinity. Because only a few species occupy the usually saline habitat of the pupfish, competition is greatly reduced. The study reported here has shown that salinity has probably posed no serious selective hazard to the pupfish. Osmoregulatory ability has apparently placed no restriction on this fish with respect to habitat.

CHAPTER V

SUMMARY

The serum osmotic pressure in freshwater-acclimated C. rubrofluviatilis was 321 ± 5.5 (S.E.) m-osmoles/Kg-water. Saltwater-acclimated fish had a serum osmotic pressure of 378 ± 7.5 (S.E.) m-osmoles/Kg-water. Acute transfer from freshwater to saltwater showed that the pupfish could acclimate to increased salinity within eight hours. During acclimation to saltwater the serum osmotic pressure reached a peak after four hours of 512 ± 20.0 (S.E.) m-osmoles/Kg-water.

Serum sodium concentration in pupfish acclimated to freshwater and saltwater was 181 ± 6.2 (S.E.) mEq/l and 204 ± 3.8 (S.E.) mEq/l, respectively. Serum potassium was higher in freshwater [6.5 ± 0.6 (S.E.) mEq/l] than in saltwater-acclimated fish [4.3 ± 0.5 (S.E.) mEq/l].

Total body sodium content in freshwater-acclimated fish [95.3 ± 4.9 (S.E.) mEq/Kg] was only slightly significantly lower than total sodium in saltwater-acclimated fish [115 ± 5.6 (S.E.) mEq/Kg]. Large sodium pools present

within the fish may contribute to the high sodium values obtained as evidenced by the large sodium content of the bile. Muscle sodium content of pupfish was also high in both freshwater and saltwater. Total body water was not significantly different in freshwater and saltwater acclimated forms [79.5 ± 0.9 (S.E.) % body weight and 77.8 ± 0.5 (S.E.) % body weight, respectively].

Apparent sodium space was 30.9 ± 2.3 (S.E.) ml/100g in freshwater-acclimated fish and upon saltwater acclimation increased to 50.9 ± 2.8 (S.E.) ml/100g. Extracellular space increased slightly after saltwater acclimation [24.9 ± 1.4 (S.E.) ml/100g to 25.2 ± 0.9 (S.E.) ml/100g].

Rate of drinking in saltwater was 1.4% body weight per hour while in freshwater it was 0.2% body weight per hour. Drinking in saltwater accounted for only 14% of the total sodium turnover per hour. The turnover rate of sodium was approximately 48% of the exchangeable sodium pool per hour in saltwater-acclimated pupfish.

Upon transfer of saltwater-acclimated fish to freshwater there was an immediate decrease in sodium efflux rate. This decrease was directly related to the external sodium concentration and was not affected by chloride concentration or osmotic pressure.

Increased Ca^{++} concentration of the external medium caused a reduction of sodium efflux in all media.

In the gills of pupfish were found large eosinophilic cells which answered the description of Keys-Willmer cells. In saltwater, these cells possessed a large apical crypt. This crypt was not present in freshwater.

Osmoregulation in the pupfish was discussed in terms of findings on other fishes. It was concluded that the tolerance of a high intracellular sodium level and the ability to regulate the composition of the blood serum were the most effective osmoregulatory mechanisms of C. rubrofluviatilis.

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