AN ABSTRACT OF THE THESIS OF

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Title: OSMOTIC AND IONIC REGULATION IN THE EARTHWORM LUMBRICUS TERRESTRIS

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Earthworms are capable of osmotic and ionic regulation while living in a dilute balanced salt solution. When transferred from soil to pond-water (PW) their body weight increases by 15 percent due to a net uptake of water. This results in an initial dilution of the coelomic fluid (CF). Subsequently, Na and Cl are accumulated in net amounts returning the CF ionic concentration to the same level as when the animals were in soil (75 mM Na/l, 47 mM Cl/l).

Water is taken up across the skin. Although worms in PW do drink, the amount is minimal. In a steady state, the rate of water uptake and loss must be equal. Water is voided as a hypo-osmotic urine from the nephridia. The clearance of Inulin and Dextran from the CF is about 75 µl/10 g-hr. Since some water is probably re-absorbed from the nephridia, this gives a maximum estimate of urine excretion. Worms living in PW use their gastro-intestinal

tract as an auxillary excretory organ. Fluid excreted from the rectum is hypo-osmotic and hypo-ionic to the CF. It is hypothesized that fluid entering the esophagous, at the level of the calciferous gland, is an ultrafiltrate of the blood. Chloride is reabsorbed from the esophageal fluid in exchange for HCO_3 ions. The esophageal fluid enters the crop. Fluid in the crop is similar in composition to the CF except that the Cl concentration is lower (2 mM/l). As the fluid passes down the intestine Na is reabsorbed in exchange for NH_4, resulting in the formation of a dilute rectal fluid.

Earthworms living in a dilute aquatic environment accumulate Na and Cl against an elecrochemical gradient by active transport across the skin. Sodium and Cl are transported independently. To maintain electrical neutrality, Na is probably exchanged for NH_4 or H ions and Cl exchanged for HCO_3. Kinetic analysis of the Na transport system for PW acclimated worms indicates a V_m of 1.1 μeq Na/10 g-hr and a K_s of 1.6 mM Na/l. These values suggest the epithelium is less permeable to Na than in most fresh-water animals.

The ion transport systems are delicately regulated. A dilution of the CF increases the rate of active transport of both Na and Cl. Sodium efflux is unchanged but the Cl efflux is reduced. The mechanism of regulation is unknown; however, neuroendocrine mediation has been implicated.

It is concluded that earthworms can adapt to an aquatic environment. However, they do possess some characteristics which distinguish them from truely fresh-water animals. The use of the gastro-intestinal tract for producing a dilute fluid excreta is unique. The relative low skin permeability to Na results in a Na transport rate much lower in magnitude than fresh-water organisms of comparable size.

Osmotic and Ionic Regulation in the Earthworm *Lumbricus terrestris*

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

OSMOTIC AND IONIC REGULATION IN THE EARTHWORM LUMBRICUS TERRESTRIS

INTRODUCTION

Oligochaetes evolved from polychaetes, presumably in a marine habitat, yet few species of oligochaetes have remained in this habitat (Barnes, 1963). The order of Oligochaeta, of which Lumbricus terrestris is a member, contains families occupying both fresh-water and terrestrial habitats. Lumbricus normally lives in moist soil. However, it is exposed to seasonal changes in their environment which create a spectrum of osmo- and iono-regulatory problems.

Animals living in the terrestrial habitat face the problems of dehydration and maintaining the proper internal ion balance. Major routes of water loss are evaporation, and fecal and nephridial excretion. Earthworms have no specialized respiratory epithelia so gas exchange occurs across the skin. This relatively large, exposed surface is potentially a major route of water loss. However, the rate of water loss across the skin is probably greatly reduced because of the high relative humidity of the underground microhabitat of earthworms. Another adaptation that minimizes the problem of dehydration is a remarkable resistance to desiccation(Adolph, 1943). Roots (1956) has shown that Lumbricus can withstand the

loss of 70 percent of its total body water over a 20 to 24 hour interval. Jackson (1926) indicated earthworms could only survive a water loss of 50 to 59 percent if the time of desiccation was shortened to five to nine hours. Measurements of the cross-sectional area of histological sections indicated that most of the water loss was from the coelomic fluid. Temperature is an important factor since heat death for Lumbricus occurs at 28°C (Wolf, 1938). There is no information on the magnitude of excretory water loss or the degree to which it can be regulated while earthworms are living in moist soil. Presumably, under conditions of limited water supply, excretory water loss can be reduced but not entirely eliminated. The ability of worms to change the fraction of ammonia and urea excretion is potentially an important mechanism for water conservation (Laverack, 1963).

Little is known about the exchanges of water and ions between an earthworm and soil water. It is unknown what fraction of soil water is available for exchange or how changes in the composition affects these animals. Although studies of this nature are important in understanding the ecological physiology of the animals, research is difficult since adequate controls are hard to maintain.

Practically all existing data on earthworm hydro-mineral metabolism are derived from worms under fresh water conditions. This offers the advantage of obtaining basic osmo- and iono-regulatory

information under relatively simplified conditions. This environment is not entirely foreign to earthworms. Periodically, during heavy rainfall, the soil may become saturated with water, turning the terrestrial habitat into an aquatic habitat.

Fresh-water animals must cope with an entirely different set of problems. The body fluids are hyper-osmotic to the medium and water entering the animal osmotically must be removed (Ramsay, 1949, a,b). Since animal body fluids are hyper-ionic to fresh water, they must regain salts lost by diffusion or excretion (Krogh, 1939; Lockwood, 1964; Kamemoto, 1964). At the present time, no systematic study has been conducted to determine how earthworms maintain an osmotic and ionic steady state while living in fresh water.

Earthworms are capable of surviving in fresh water. Roots (1956) has maintained worms in water-saturated soil for almost a year and in running tap water, unfed, for several months. Death was probably due to starvation (Laverack, 1963; Roots, 1956).

Reports are conflicting about weight changes occurring when earthworms are transferred from soil to fresh water. Frequently, they increase in weight by 15 to 30 percent during the first hours in water and this weight is maintained constant when handled carefully during the weighing procedure (Adolph and Adolph, 1925; Maluf, 1939; Adolph, 1927). The increase in weight is due to a net accumulation of water. However, some worms show an initial increase in

weight followed by a decline, often below the initial weight (Adolph, 1927; Maluf, 1939; Wolf, 1940; Bahl, 1945). The water uptake and retention is probably the normal response to entry into fresh water (see below). There are several reasons for the subsequent weight loss.

Wolf (1940), in a systematic study of the effects of handling, demonstrated that if worms which had been in fresh water for 24 hours were blotted on filter paper to remove adherent moisture they would lose 12 to 15 percent of their body weight. This would reduce the body weight to the same level as when the animals were in soil. Heavy metals have also been shown to be lethal for earthworms. Tap water or distilled water from tin or copper coated pipes caused about 50 percent mortality within 24 hours (Brink and Rietsema, 1949). Finally Wolf (1940) showed that exposure to glass distilled water caused weight losses and mortality for some worms within three days.

The integument and the gut are two possible routes for water entry in earthworms. The primary route of osmotic water uptake for worms in tap water has been shown to be the integument (Maluf, 1939; Bahl, 1945). Ligation of the anterior and posterior of earthworms taken from soil and transferred to tap water resulted in the usual 15 to 30 percent weight gain (Maluf, 1939). The gut plays a minor role in water uptake. Dissection of the gut of worms

immersed in a solution of phenol red revealed no dye (Maluf, 1940). He concluded that earthworms in fresh water do not drink.

The rate of osmotic uptake of water by an earthworm immersed in tap water is approximately 60 percent of its body weight per day (Wolf, 1940). This rate was estimated by handling the animal causing a weight loss, then after it reached a constant weight returning it to water. The rate of weight gain during the first two hours was used to calculate the osmotic uptake of water. Presumably the handling caused expulsion of water from any storage site. It is assumed that only a minimum amount of water was voided during the first two hours after return to tap water and there were no skin permeability changes to water. In a steady state water must leave at the same rate as it is taken up.

Water is voided as a urine hypo-osmotic to the body fluids (Bahl, 1945, 1947; Ramsay, 1949, a, b; 1954). Bahl (1945) has obtained an estimate of the rate of urine formation in the Indian earthworm, Pheretima posthuma. Placing several worms, which had been acclimated to tap water, in a sloped glass container on one side of a partition, he was able to collect the fluid which passed under the partition to the other compartment. Using this method he found an average rate of fluid exuded from the nephridiopores and gut to be 45 percent of their body weight per day. At least part of the fluid from the gut was derived from urine since this species has

nephridia emptying into the gastro-intestinal tract. Although this fluid is subject to alteration by being in contact with the worm's epithelium, gut residue and mucous as well as possible evaporation, the rate of formation is in the range of the uptake of water. The fluid collected had an osmolarity of 27 to 32 mOsm/l which is significantly less than the coelomic fluid (160 mOsm/l). Traces of Na, K and Ca were detected but the concentrations were not determined. The chloride concentration of fluid collected from worms in distilled water for six days was 1 mM Cl/l. Ramsay (1949, a,b) confirmed both the osmotic pressure and the chloride concentration on urine samples collected from nephridia directly.

Recently, Boroffka (1965) has studied the mechanism by which the nephridia of Lumbricus forms a hypo-osmotic urine. Her experiments show that Na is actively transported across the nephridial epithelium from the lumen to blood. The rate of active Na transport is similar to that found in vertebrate epithelia involved in Na transport (0.9×10^{-5} $\mu eq/mm^2$ -sec). Measurements of the short-circuit current indicated chloride follows sodium passively. The urine remains hypo-osmotic because of the relative impermeability of the distal portion of the nephridium to water.

The alimentary tract of L. terrestris may also play a role in water regulation. Early studies indicated that the loss of weight caused by handling was from fluid expelled from the mouth and

rectum (Adolph, 1927; Maluf, 1939). However, upon re-examination, Maluf (1940) concluded that since the anterior gut fluid had a higher osmotic pressure than the coelomic fluid, the fore gut was of no importance in the formation of a hypo-osmotic fluid necessary to maintain water balance. Wolf (1940) also considered the gut of no importance to water balance. Worms that had their anterior and posterior ends ligated, while acclimating to tap water, lost weight when handled and blotted. The fluid was observed to be voided from the nephridiopores.

Although the urine is hypo-osmotic to body fluids, it still contains significant quantities of ions. Furthermore, any ion losses via the gut or by diffusion across the skin increase the problem of maintaining an ionic steady state. Yet Maluf (1940) and Kamemoto et al. (1962) have observed that earthworms in tap water for seven to sixteen days have a body fluid ionic composition essentially identical with animals in moist soil. To maintain an ionic steady state, L. terrestris must be able to accumulate salt. A feeding animal can recover some ions from its food, but since worms kept in water are not fed this route cannot be used. The site of salt accumulation is probably the integument. The ability of animal epithelia to actively transport or accumulate salts has been well documented (Krogh, 1939; Potts and Parry, 1964; Potts, 1968), but data on oligochaetes are fragmentary.

Before the use of radioactive isotopes, the ability of animals to take up salts against a concentration gradient was studied by salt depleting the animal in distilled water prior to exposure to dilute salt solutions. Using this technique, Krogh (1939) was able to show that a variety of fresh-water animals (frog, fish and mollusks) could absorb salt (chloride) from dilute media. This method was used by Brink and Rietsema (1949) to demonstrate the net uptake of Cl from tap water by starving earthworms.

With the advent of radioisotopes and refined methods for measuring chemical concentrations, electrical potentials and currents, it became possible to determine which ions are actively transported by epithelia (Ussing, 1949). Ussing and Zerahn (1951) conclusively demonstrated that active sodium transport was responsible for the generation of the short-circuit current in the isolated frog skin. Tercafs (1965) has applied the same technique to skin stripped from the body muscles of Lumbricus. He concluded that Na is actively transported by the skin from the external solution to the internal solution. Chloride follows passively.

In vitro skin studies are conducted with balanced salt solutions of equal composition bathing both sides of the skin. However, worms living in dilute solutions have different concentration and electrical gradients influencing the movements of ions. There have been no systematic studies on the mechanisms employed by

Lumbricus in maintaining an ionic steady state. Isolated skins are separated from control mechanisms which may modify the transport systems. Recently, Kamemoto (1964) has implicated some factor in the brain of worms which controls salt and water balance in earthworms. The mechanism of action is unknown.

The purpose of this thesis is to examine the mechanisms by which the earthworm, Lumbricus terrestris, maintains an osmotic and ionic steady state while living in a dilute aquatic environment. Extensive use of radioisotopes has afforded information on the dynamic nature of the transport mechanisms involved in achieving homeostasis.

MATHEMATICAL TREATMENT

The relationship between the unidirectional movements of ions (fluxes) and the net exchange may be represented by the following equation

$$M_n = M_i - M_o \qquad (1)$$

where M_i is the unidirectional influx, M_o the unidirectional efflux and M_n the net flux. The unidirectional fluxes are measurable with the aid of radioactive isotopes and the net flux can be determined by chemical analysis. Thus if one of the fluxes (M_i or M_o) is measured the other can be calculated from equation 1.

Equations for calculating the unidirectional movement of an ion have been derived by Jørgensen et al. (1946). The derivation is for influx but the efflux can be found similarly.

If the isotope is added to the bath the rate of disappearance of radioactivity, $-dc^*/dt$, is a function of the rate of movement of the ion, dA/dt, and the specific activity of the bathing medium, c^*/cV. Thus:

$$dc^*/dt = (-dA/dt)c^*/cV \qquad (2)$$

where c^* is the total radioactivity (counts/minute), A is the total amount of the ion under study (microequivalents), c is the

concentration of the ion in the bathing solution (μeq/ml) and V is the bath volume (ml).

Equation 2 is based on several important assumptions which allow simplification and evaluation. The mechanism of ion uptake does not distinguish between stable and unstable isotopes; the radioactivity is confined predominantly to one compartment so that back flux of the isotope is negligible; the influx of the ion is constant over a short time interval so that dA/dt becomes the influx, M_i.

From these assumptions, equation 2 can be rewritten as

$$dc^*/dt = M_i c^*/A \tag{3}$$

depending upon the conditions, two solutions can be obtained when equation 3 is integrated. (I) The animal is in a steady state so that the amount of the ion in the bathing medium remains constant (A = constant). (II) The animal is not in a steady state and the amount of ion in the bath either increases or decreases.

For condition (I) we can rearrange equation 3

$$\int dc^*/c^* = (-M_i/A) \int dt \tag{4}$$

integrating gives

$$\log_e c^* = M_i t/A + k \tag{5}$$

where k is the integration constant, which can be evaluated if c* is designated as c_o^* at time zero

$$M_i = \log_e(c_o^*/c^*) \cdot A/t \tag{6}$$

By measuring the change in counts and the total amount of the ion in the bath over an interval of time, M_i can be determined.

During an experiment where the amount of ion changes (II), the time interval must be sufficiently short so that the net change in the ion proceeds at a constant rate (M_n). The change in the amount of the ion can be expressed:

$$A = A_o - M_n t \tag{7}$$

differentiating this equation with respect to time

$$dA/dt = -M_n \quad \text{or} \quad dt = -dA/M_n \tag{8}$$

inserting equation 8 into 4

$$\int_{c_o^*}^{c^*} dc^*/c^* = (M_i/M_n) \int_{A_o}^{A} dA/A \tag{9}$$

integrating this equation

$$M_i = \frac{\log_e (c_o^*/c^*)}{\log_e (A_o/A)} \cdot M_n \tag{10}$$

Although the equations for M_i are written for total number of counts and total amount of the ion, in evaluating the equations the concentrations are used. This simplifies computation and is justifiable where the ratios are used since volume is cancelled out of the equation. These equations were programmed for a computer and the fluxes expressed as μeq/10 g-hr.

Movement of material may be either by passive diffusion down established gradients or by active transport. Active transport is defined as the movement of material against the electrochemical gradient at the expense of metabolic energy (Ussing, 1949; 1954).

Passive diffusion can be determined from the following consideration. If the inward and outward permeability coefficients for any ion are the same, then the passive diffusion of ions should obey the flux-ratio equation derived by Ussing (1949)

$$M_i/M_o = (c_o/c_i) \exp (zFE/RT) \qquad (11)$$

where the subscripts i and o refer to the inside and outside compartments, M is the flux in microequivalents cm^{-3}; c is the ionic concentration in microequivalents cm^{-3}; z is the valency of the ion; F is the Faraday, 96500 joules $volt^{-1}$ $mole^{-1}$; E is the electrical potential difference across the membrane separating the two compartments with reference to the outside, in volts; R is the gas constant in electrical units of 8.3 joule $degree^{-1}$ $mole^{-1}$ and T is

the absolute temperature in degrees. The flux-ratio equation indicates that passive diffusion is proportional to the electrochemical gradient. If the observed flux ratio is not in agreement with the calculated flux ratio from equation 11, then it may be concluded that the material is actively transported.

MATERIAL AND METHODS

Collection and Care of Animals

The earthworms used in this study, Lumbricus terrestris, were obtained from a local bait dealer. The animals were stored in plastic containers filled with a leaf mulch soil and maintained in a constant temperature $14 \pm 1^{\circ}C$. All of the experiments, except where noted, were conducted in the same room. The soil moisture was not controlled and water was added as needed. The photoperiod was usually 14 hours and was not changed with the season. The size of animals used in this study ranged between one and ten grams but usually averaged about three grams.

Artificial Pond Water

To standardize the environmental conditions most of the animals were transferred to an artificial pond water (PW) for at least a week prior to use. The composition of PW was 0.50 mM NaCl, 0.05 mM KCl, 0.40 mM $CaCl_2$ and 0.20 mM $NaHCO_3$ per liter of water. Groups of 12 to 20 worms were maintained in the plastic containers with about two liters of continuously aerated PW.

Experimental Conditions

Most experiments were conducted on groups of eight animals

from the same population. The animals were placed individually into pint containers having a small volume of the experimental medium (usually 25 ml). For experiments employing radioactive isotopes the bathing medium was aerated and stirred by bubbling water saturated air into the bath. Volumetric samples of the bath were removed without replacement at timed intervals. Samples for chemical analyses were usually diluted. Undiluted bath samples were used for radioactivity assay.

Coelomic and Crop Fluid Samples

Coelomic fluid (CF) was collected from worms without the aid of an anesthetic, however, the animals were immobilized by packing in ice. The CF samples were collected by inserting a small, pointed capillary tube through the body wall into the coelom. The CF was transferred to Beckman microfuge tubes for storage. To obtain crop fluid, the crop was exposed by cutting through the skin. Adherent CF was removed by blotting and a pointed capillary tube inserted into the crop lumen. The fluid was stored in Beckman microfuge tubes. Prior to use, each sample was centrifuged to remove particulate matter. If the samples were not to be analyzed within a few hours, they were stored at -20^{o}C, otherwise they were refrigerated at 3^{o}C.

Transepithelial Electrical Potentials

An in vivo method was used to measure the transepithelial electrical potentials (TEP) generated by earthworms. A mid-lateral incision into the coelomic cavity was made and a small polyethylene bridge filled with three percent agar-Ringer was inserted. The other end of the bridge was placed in a calomel electrode. The animal was supported with forceps keeping the incision above the bathing solution to avoid short-circuiting. One end of a second bridge was placed in the bathing solution and the other end placed in a reference calomel electrode. The two electrodes were connected to a recording potentiometer. The basic bathing solution was 1 mM/l K_2SO_4 to obtain a conducting medium. Solutions were added to the bath to give the required ionic composition and concentrations. Samples of the bath were taken for electrical asymmetry determination and ion analyses.

Coelomic Fluid Clearance

The rate of removal of selected material injected into the CF of worms was measured. Clearance of carbon-14 labeled Inulin, high molecular weight Dextran or Glycine was measured on individual animals. Solutions made up in Ringers were injected into the coelomic cavity then the animals were placed in PW and allowed one

hour to distribute the isotope throughout the CF. After equilibration, each animal was rinsed, weighed and transferred to 10 ml of PW in a plastic container. The containers were placed in a water bath maintained at 15 ± 0.5°C and gently shaken (90 times per minute). One ml samples were removed from the bath at hour intervals for isotope assay. At the termination of the experiment a coelomic fluid sample was collected and a known volume (10µl) counted to determine the count rate per volume of CF. Knowing the rate of counts appearing in the bath and the CF activity, the volume of CF being cleared of the isotope per hour could be determined. The data were standardized to a 10 g animal.

Chemical Analyses

Specific ion determinations of bathing media and CF samples were performed on diluted samples. Coelomic fluid which had been stored frozen was brought to room temperature (23°C) prior to use. Sodium and potassium concentrations were determined using flame photometry (precision ±1%). Chloride concentration was measured with an Aminco-Cotlove chloridometer (precision ±1%). Total solute was determined on undiluted samples using a Mechrolab vapor pressure osmometer (precision ±2%).

Total Na, K and Cl contents of earthworms were also determined. The animals were dried to a constant weight at 105°C. For

Na and K determination the carcasses were digested with hot concentrated HNO_3. The digest was diluted for flame photometer analysis. The HNO_3 did not interfere with Na or K determination. For total Cl determination the dried animals were ground to a powder and extracted with distilled water for 12 to 24 hours at $3^{o}C$. This technique gave 100 percent recovery of added Cl indicating minimal titration interference from extracted organic material.

Isotope Analysis

Most of the experiments involving ^{22}Na or ^{36}Cl were assayed for radioactivity by pipetting samples onto 2 ml aluminum or steel planchets. Water and two drops of 1 percent Tween 80 were added to ensure uniform layering while the planchets were dried on a hot plate. If the sample contained acid, steel planchets had to be used. Steel planchets back-scatter 18 percent more radiation than aluminum and correction was necessary when the planchets were mixed within an experiment. Planchets were counted on a Nuclear-Chicago gas-flow automatic geiger counter. Most of the ^{14}C samples were pipetted into 20 ml screw cap vials for liquid scintillation counting. Ten ml of a scintillation fluor was added (2 l toluene, 1 l Packard Triton X-100, 100 mg POPOP, 4 g PPO) and the water content was adjusted to 1 ml by adding distilled water. The samples were thoroughly mixed and allowed to stand in the darkened sample

changer at least an hour before counting. The detector was a Nuclear-Chicago ambient temperature scintillation counter. An automatic external standard was used to detect changes in quench. Counting precision for either system was usually greater than $\pm 2\%$.

Statistical Analysis

All values for populations of animals are presented as the mean $\pm$ one standard error of the mean. Differences between means were considered significant if $P < 0.05$.

RESULTS

Effects of the Ionic Composition of the External Medium

Earthworms in moist soil are subjected to uncontrolled variations in external ionic concentrations and water content. Two different analyses performed on water extracts of air dried soil (105°C) gave the following values: 2.3 to 4.4 mM Na/l, 9.1 to 14.3 mM K/l, 0.9 to 3.9 mM Cl/l and 62 to 70 percent water content. These values are not necessarily the extremes.

As indicated in the introduction, basic osmo- and ionoregulatory information for earthworms can be obtained from studies conducted on worms in an aquatic environment. There are several experimental advantages gained under these conditions. Samples are easily obtained and ionic concentrations determined; the ionic composition can be controlled; and contamination from radioisotopes is minimized and waste disposal problems are reduced.

Lumbricus is able to live for extended periods of time in an aquatic habitat. Although others have demonstrated the influence of external salinity on coelomic fluid osmolarity and Cl concentrations, the changes in Na concentration, the predominant CF cation, have not been studied. Thus a study was conducted to determine the effects of external ionic concentrations on the CF ionic

composition. Groups of six to eight worms were transferred from soil to aerated NaCl solutions ranging in concentration from 5 to 150 mM/l. Each group was allowed to acclimate for 10 to 13 days before sampling their coelomic fluid. There was no mortality. Figure 1 shows the results. Sodium concentration is added to the Cl concentration in each column. Earthworms are hyper-regulators with respect to Na and total solute in solutions below 78 mM NaCl. Above 78 mM NaCl the CF remains slightly hyper-osmotic but the Na concentrations are essentially equal to the bath. Chloride is hyper-regulated in solutions below 40 mM NaCl but hypo-regulated above this. In fact, chloride remains essentially constant until the bath concentration exceeds 78 mM/l. Throughout the entire range, Na and Cl account for most of the CF total solute. There is a slight increase in the total solute above that contributed by Na and Cl in the 126 and 150 mM NaCl acclimated animals. The source is unknown. Potassium concentration is about 3 mM/l and remained constant under all of the conditions.

While conducting the above experiment, it was noted that worms would not survive exposure to dilute NaCl solutions (< 1 mM) for more than seven to 14 days. To study the effects of ions in dilute media on survival, groups of 10 worms were placed in containers having various combinations and concentrations of ions found in fresh water. Table 1 presents the weight changes and survival of

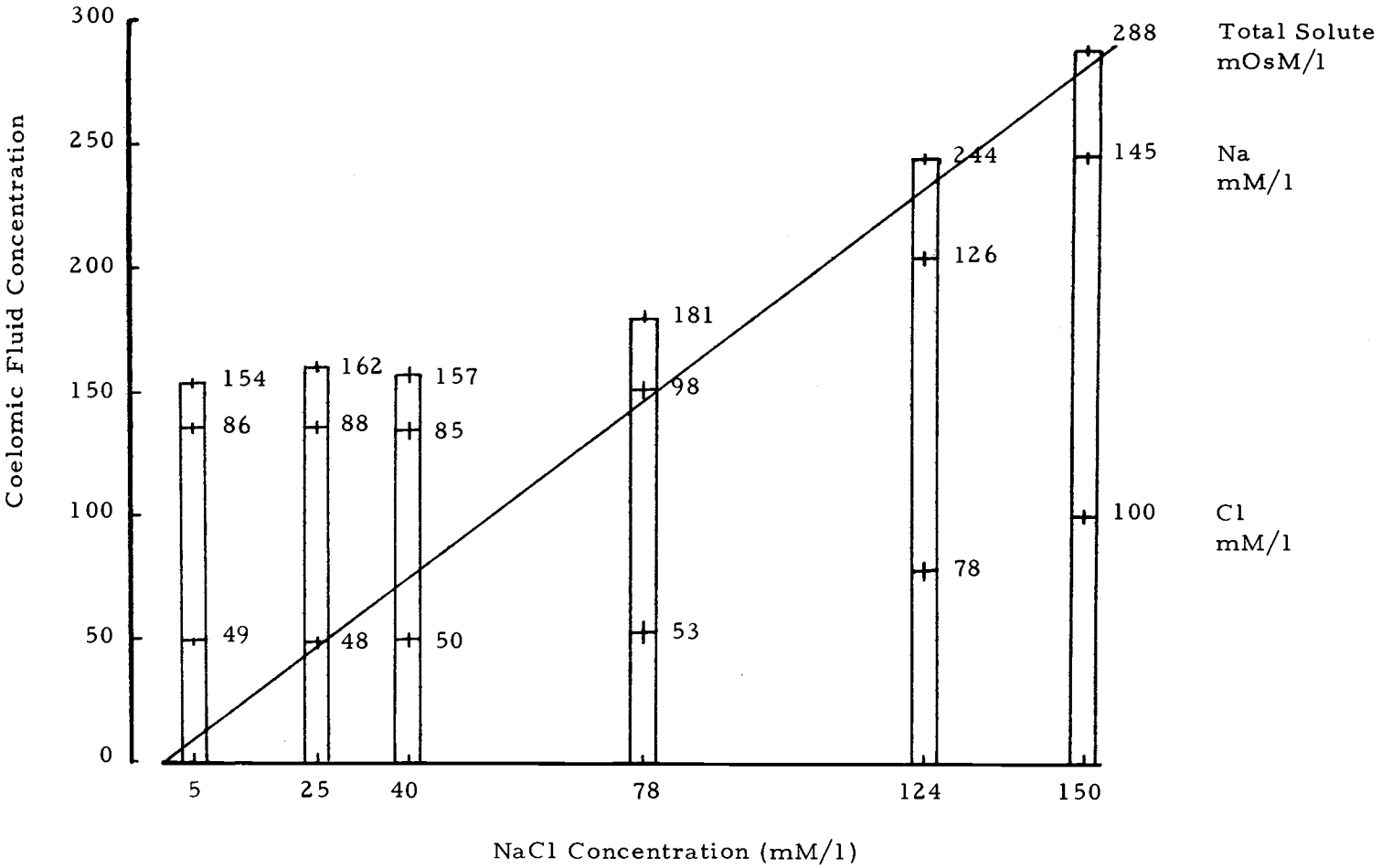

Figure 1. Coelomic fluid composition of worms acclimated to different NaCl solutions. Na was added to the Cl concentration. The numbers adjacent the bars represent the mean value; the vertical lines are ± S.E. The diagonal represents iso-osmoticity.

worms under these conditions.

Table 1. Effects of ion combinations on body weight and survival of earthworms. The weights were based on the initial weight of the animal in soil (B_o).

Ion Concentrations	% B_o 9 Day	Survival 13 Day	Survival 16 Day
1.0 mM NaCl	96.3	8	0
1.0 mM NaCl + 0.05 mM $CaSO_4$	112.0	10	10
1.0 mM NaCl + 0.1 mM $CaSO_4$	109.5	8	8
0.8 mM NaCl + 0.1 mM Na_2SO_4	95.0	8	0
1.0 mM NaCl + 0.5 mM KCl	107.0	10	10
1.0 mM NaCl + 0.12 mM K_2SO_4	119.0	9	0
Pond Water	115.0	10	10

All of the animals surviving 16 days gained weight but none gained as much as the worms in PW (115 percent of the animals weight in soil, B_o). Survival was high in the solutions containing $CaSO_4$ or KCl with NaCl. Apparently Ca or an inequality of Na and Cl promote survival in dilute solutions. The ionic composition of the artificial PW is given in the Methods. PW is a balanced salt solution containing the ions which, in combination with NaCl, promote earthworm survival in dilute media.

Acclimation to Pond Water

Worms survive in pond water for months. A comparison of the ion content and coelomic fluid composition between soil and PW acclimated worms (> 7 days in PW) is given in Table 2. The differences in water content and total Na are significant ($P < 0.05$). The differences in K and Cl content are not significant. No difference was observed between the two groups with respect to CF Na, K or Cl concentrations or total solute.

Table 2. Total ion composition and coelomic fluid concentrations for worms acclimated to soil or PW.

Measurement	Units	Soil	Pond Water
Water Content	ml/10g	8.4 ± 0.1 (18) [1]	8.8 ± 0.0* (22)
Total Na	μeq/10g	235 ± 3 (8)	373 ± 15 * (12)
Total K	μeq/10g	345 ± 15 (8)	365 ± 15 (12)
Total Cl	μeq/10g	142 ± 13 (8)	174 ± 13 (12)
CF[2] Na	μeq/ml	71 ± 2 (14)	75 ± 1 (24)
CF K	μeq/ml	4 ± 0 (14)	3 ± 0 (24)
CF Cl	μeq/ml	48 ± 1 (14)	47 ± 1 (24)
CF Total Solute	mOsm/l	154 ± 2 (10)	159 ± 2 (15)

[1] Mean ± Standard error of the mean (N)

[2] Coelomic fluid

* Significantly different ($P < 0.05$)

It is apparent that some ionic adjustment must occur in worms transferred from soil to an aquatic environment. Figure 2 illustrates the changes occurring in the CF ionic concentrations and body weight during the first three days in PW. Worms were removed from soil, weighed and placed in individual containers with 50 ml of PW. After specified intervals the animals were removed, weighed and the CF sampled. Within 24 to 48 hours the body weight stabilized at 115 percent above the weight of the animal in soil (B_o). The CF ion concentrations decreased within 24 to 48 hours by approximately 15 percent. However, the PW acclimated animals have the same CF concentrations as soil animals even though the water content is higher (Table 2). Apparently some time between three and seven days the CF ion concentrations are elevated and, in fact, careful examination of Figure 2 indicates this trend.

To determine the nature of the ionic readjustment in earthworms acclimating to PW, changes in the environment as well as changes in the animal must be monitored. Eight animals were placed in individual containers having 50 ml of PW. Serial bath samples were taken and the results of the ion analyses averaged. From changes in the bath ionic concentrations, the net exchanges between the worms and their environment were determined (Figure 3). The bath was replaced with fresh PW at 48 hours.

The mean body weight increased 114 percent B_o and was

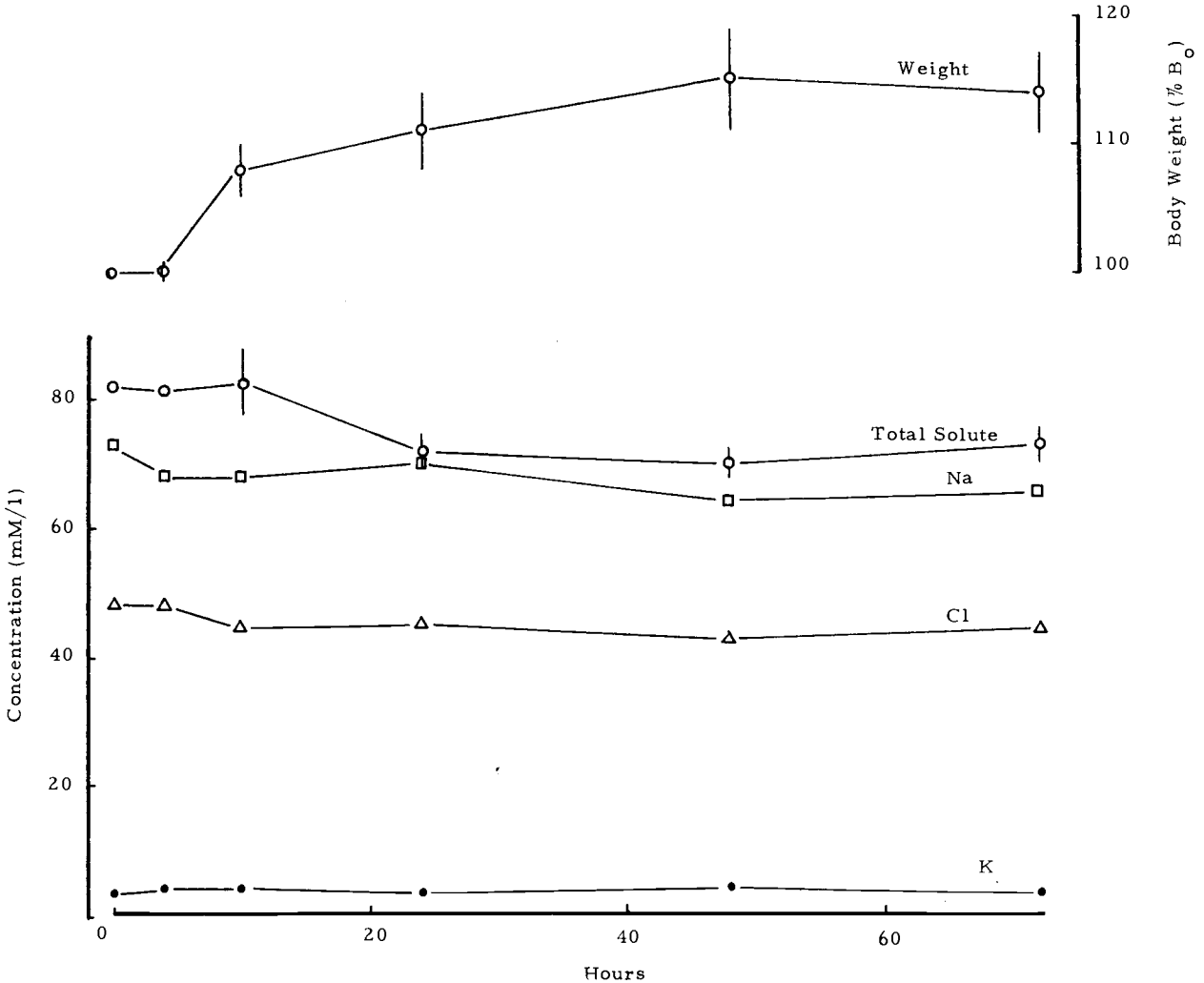

Figure 2. Changes in body weight and coelomic fluid in concentrations after transferring worms from soil to PW. Total solute is expressed as mM/l NaCl.

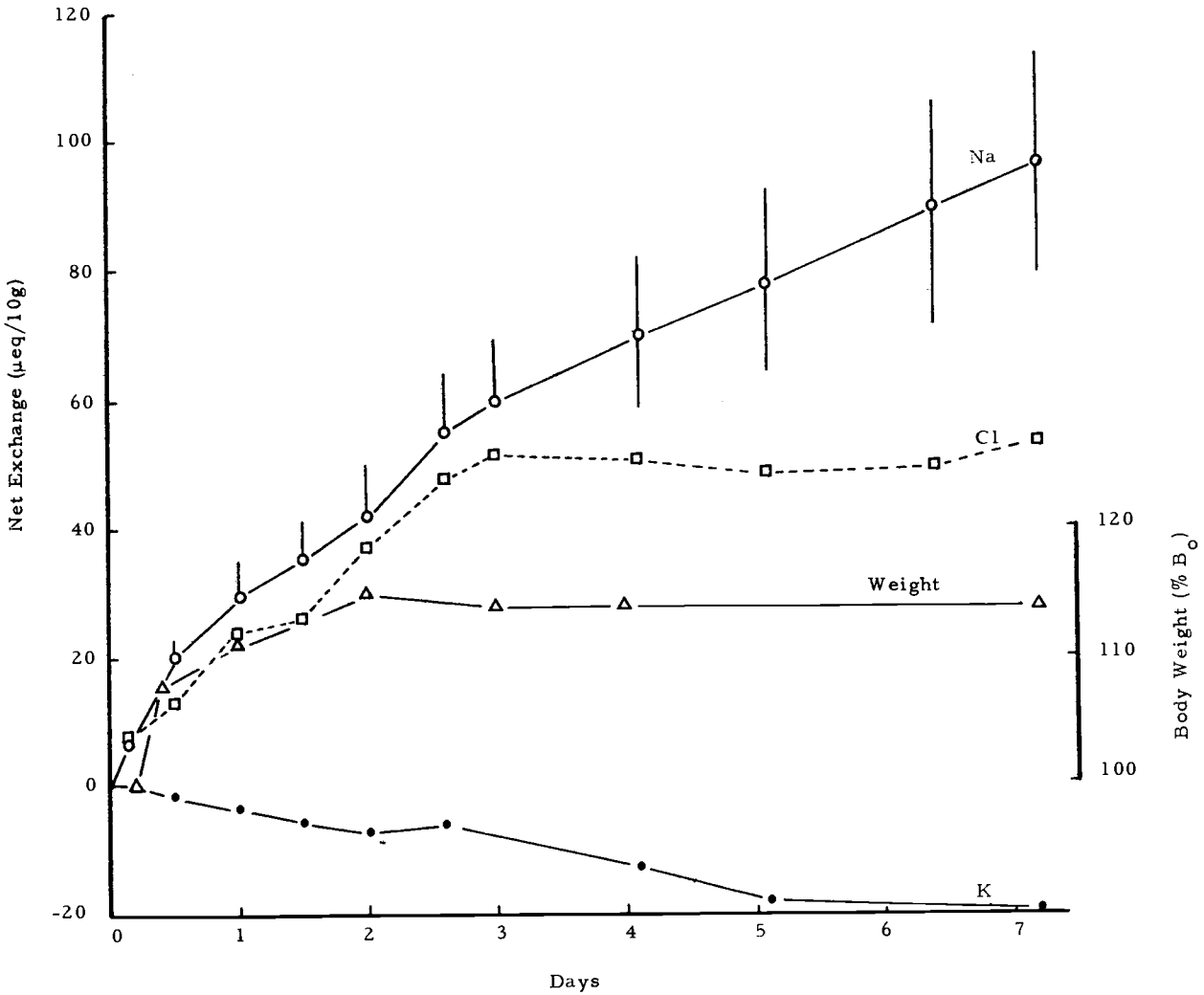

Figure 3. Net exchange of ions and water for worms transferred from soil to PW. Body weight is expressed as percent of the initial weight (B_o).

maintained constant after two days. The animals accumulated net quantities of Na and Cl from the dilute PW; they suffered a net loss of K. After three days the animals came into a Cl steady state. In this experiment Na was continually accumulated in net amounts, however, subsequent experiments have demonstrated that worms come into and maintain a steady state for Na within seven days.

These experiments indicate that upon transfer from soil to PW, earthworms take up net amounts of water which dilutes their body fluids. Subsequently, Na and Cl are taken up in net amounts and ultimately the CF ionic concentrations are restored to values comparable with those of soil animals. These results indicate the presence of a well developed system controlling the iono-regulatory mechanisms.

The total Na, K and Cl content of an animal are distributed between the extracellular and intracellular compartments. The distribution volume of an ion can be determined by dividing the total amount of an ion by the CF concentration for the respective ions. This value is called the ion space and has the units of volume (ml). The Cl space for a 10g PW acclimated worm is 3.7 ml. The Na space is 5.0 ml. Chloride is primarily confined to the extracellular space in animals (Davson, 1964) so the Cl space would be a maximum estimate of the extracellular space in the earthworm. Apparently a significant amount of Na is not in the extracellular

compartment. The Na and Cl spaces for worms acclimated to soil are 3.3 and 2.5 ml respectively. The entire Cl content of a worm is readily exchangeable with Cl-36. However, Na is only 62 percent exchangeable with Na-22 within 11 days. This indicates a significant amount of Na is "bound" or compartmentalized. When transferred from soil to PW, a 10g worm would have gained about 1.3 ml of water. This is very close to the 1.2 ml increase in Cl space observed in PW animals, indicating most of the water taken up is confined to the extracellular space when the animals finally reach a steady state. The Na space increases by 1.7 ml indicating a significant amount of Na is associated outside the extracellular compartment. Changes in the amount of Na in the intracellular compartment or "bound" are not known.

Kinetics of Water Balance

The data presented above indicate earthworms have the capacity for osmo- and iono-regulation in the aquatic environment but do not indicate the routes of water and ion exchange. There are two routes for water to be taken up: the gastro-intestinal tract and the skin. Water may be lost by a combination of two different routes: nephridial excretion and rectal excretion.

Previous attempts to determine if earthworms drink water were conducted by adding dyes to the water and visually inspecting

the gut for its presence. This technique has two disadvantages: small amounts of dye cannot be observed and the rate of water consumption cannot be quantitated. Both of these problems can potentially be circumvented by using a radioactive tracer.

Earthworms in PW have been observed to open their mouths periodically. These animals will also feed on material left in the water. Thus the gut is a potential site of water uptake. To measure the volume of fluid ingested, worms have been placed in PW with Inulin-14C for periods ranging from two hours to nine days. Bath samples were assayed for loss of radioactivity. After the animals were exposed to the isotope they were either sacrificed and samples from the gut and CF assayed for isotope or they were rinsed and placed in unlabeled PW and the appearance of radioactivity in the bath monitored.

Of four animals used for Inulin uptake, two did not change the bath activity during 41 hours whereas two reduced the activity indicating a mean drinking rate of about 100 μl bath/10g-hr. In another experiment, crop, midgut and coelomic fluids were sampled from three worms which had been in PW with Inulin-14C for nine days. The crop fluid activity ranged between 29 and 69 percent of the bath activity. The midgut fluid was between 39 and 69 percent of the bath activity but the CF did not exceed 5 percent, which is within the range of experimental error. Wash-out experiments designed to

measure the rate of appearance of Inulin-14C in an unlabeled bath were unsuccessful because of the low specific activity of the gut fluids and the dilution of isotope in the bath. Since some isotope did appear in the gut, these animals must drink some water. However, since the gut fluids did not have the same activity as the bath, drinking could not be as important as osmosis through the skin for the uptake of water. The low radioactivity in the coelomic fluid suggests minimal degradation of the Inulin during the experiment.

Measurement of the rate of nephridial excretion is extremely difficult and the validity of published values is questionable. A maximal estimate can be obtained by measuring the coelomic fluid clearance rates of certain substances. Several assumptions must be made in selecting the material to be used: it is not stored or metabolized, entry into the nephridia is unrestricted and there is no reabsorption from the nephridia. Inulin-14C (M.W. 5200) has been shown to be satisfactory in several animals. If the presumptive urine entering the nephridia is coelomic fluid entering via the nephrostome, then all substances satisfying the assumptions should give the same clearance value. High molecular weight Dextran-14C (M.W. 60,000 to 90,000) should be satisfactory whereas Glycine-14C should not. Clearance values for these substances have been determined for worms acclimated to soil and/or PW (Table 3).

Table 3. Coelomic fluid clearance rates in earthworms.

Substance	N[1]	n[2]	Clearance µl/10 g-hr
Soil Acclimated			
Inulin	4	16	10.0 ± 2.1
Inulin	6	23	83.0 ± 18.9
Pond Water Acclimated			
Inulin	7	28	70.7 ± 10.7
Dextran	5	20	75.8 ± 19.4
Glycine	5	20	10.3 ± 3.8
Inulin[3]	4	8	86.2 ± 24.5
Dextran[3]	3	8	53.2 ± 10.8

[1]Number of animals.

[2]Number of observations.

[3]Experiment performed in PW iso-osmotic with the coelomic fluid by sucrose.

Worms acclimated to soil were found to separate into two groups with respect to Inulin clearance. Of 10 animals studied, six had clearance rates eight times higher than the rest and they were treated separately in the table. Pond-water acclimated worms clear the CF of Inulin and Dextran at similar rates which are indistinguishable from the Inulin clearance rates of the worms acclimated to soil.

Glycine, however, is cleared from the CF at 1/7 the rate of Inulin or Dextran indicating reabsorption. The low Inulin clearance rate observed in the four soil animals indicates that all worms do not respond to the osmotic entry of water equally. It is not known if the 10 μl/10g-hr clearance rate is the normal value for worms in soil or if these animals were anti-diuretic. Clearance of Inulin and Dextran from the CF of PW acclimated worms did not change even when the worms were in a sucrose solution, iso-osmotic with the CF for six hours. Apparently the rate of water entering the animal does not directly control its rate of elimination.

The amount of water reabsorbed by the nephridia could not be determined so the actual volume of urine is unknown. It is probably somewhat less than the 75 μl/10 g-hr average CF clearance value. Samples of nephridial fluid were not collected so no urine analyses have been performed in this study.

Earthworms maintained in PW have been observed to void a fluid excreta from the anus. The rate of fluid elimination by this route has not been measured, although samples have been obtained from the rectum. Neither Inulin nor Dextran in the CF was voided through the rectum during the five hours of the clearance measurements.

Kinetics of Ion Balance

Earthworms acclimated to PW usually maintain a steady state with respect to Na and Cl. The unidirectional fluxes and net flux for these ions are listed in Table 4. The differences between the influx and efflux for each ion are not significant. Part of the difference in M_i between Na and Cl is due to the higher Cl concentration in PW.

Table 4. Mean Na and Cl fluxes over a 48 hour interval for worms acclimated to PW.

Ion	N	μeq/10 g-hr		
		M_i	M_o	M_n
Na	11	0.25 ± 0.06	-0.32 ± 0.08	-0.07 ± 0.05
Cl	15	0.88 ± 0.10	-0.90 ± 0.14	-0.02 ± 0.08

Solutes may be lost by a combination of three different routes: rectal excretion, nephridial excretion and diffusion through the skin. Samples of the rectal fluid were obtained by inserting a capillary tube into the rectum. Solid material was separated by centrifugation. The Na, Cl and total solute concentrations of rectal fluid are significantly lower than the CF (Table 5). The CF ion concentrations for this population of worms are lower than the values listed in Table 2; the cause is unknown. No nephridial samples were analyzed but

from published data (Ramsay, 1949 a, b) it is probably quite similar to the rectal fluid. Fluid was never observed to be expelled from the mouth. Detection of this fluid would be facilitated by its brown color. Analyses of the crop fluid indicate it has about the same Na, K and total solute concentrations as the coelomic fluid, however, the Cl concentration is considerably lower at 2 mM/l (Table 5). The nature of the unidentified crop solute is not known. Since drinking is minimal in PW acclimated worms, most of the crop fluid must enter by way of the blood or CF.

Table 5. Coelomic fluid, crop fluid and rectal fluid composition of PW acclimated worms.

	mM/l			mOsm/l
Fluid	Na	K	Cl	
Coelomic	66 ± 3 (10)	3 ± 0 (10)	42 ± 2 (10)	151 ± 4 (18)
Crop	56 ± 3 (8)	4 ± 0 (7)	2 ± 0 (8)	153 ± 4 (16)
Rectal	7 ± 2 (7)	3 ± 0 (3)	13 ± 5 (9)	37 ± 11 (3)

The average earthworm has 280 nephridia and since they could not be effectively occluded, the diffusion of ions across the skin was not measured directly. An indirect method of partitioning renal and extrarenal ion loss is to measure the M_o with and without the osmotic entry of water. Addition of sucrose to PW making the bath

iso-osmotic with the worms body fluid stops the osmotic uptake of water. An experiment measuring fluxes before and after addition of sucrose indicated no change in Na efflux within 17 hours. Either the renal component is very low, or the renal excretion is relatively independent of water uptake (see above).

To maintain a steady state the accumulation of ions must balance the loss. There are two potential routes for ion accumulation: the gut and the skin. Several factors discount the gut as a significant source of ion uptake from the environment. In the experiments studying drinking rates, worms were not found to have the same isotope activity in the gut as the bath. Yet PW animals having a M_i of 0.3 μeq Na/10 g-hr should have turned over the Na in the bath at least twice in the nine days. Since the rectal fluid has been shown to contain a higher concentration of ions than found in PW, any drinking would aggravate the rate of ion loss. Thus the skin must be the major route of ion transport.

To demonstrate active transport of an ion, it must be shown that the ion is accumulated against an electrical gradient as well as a concentration gradient. The transepithelial electrical potential (TEP) for two representative worms acclimated to PW are represented in Figure 4. The worms were exposed to NaCl and Na_2SO_4 during two consecutive runs. The sign of the TEP is with respect to the inside of the animal. The TEP was recorded after 10 minutes

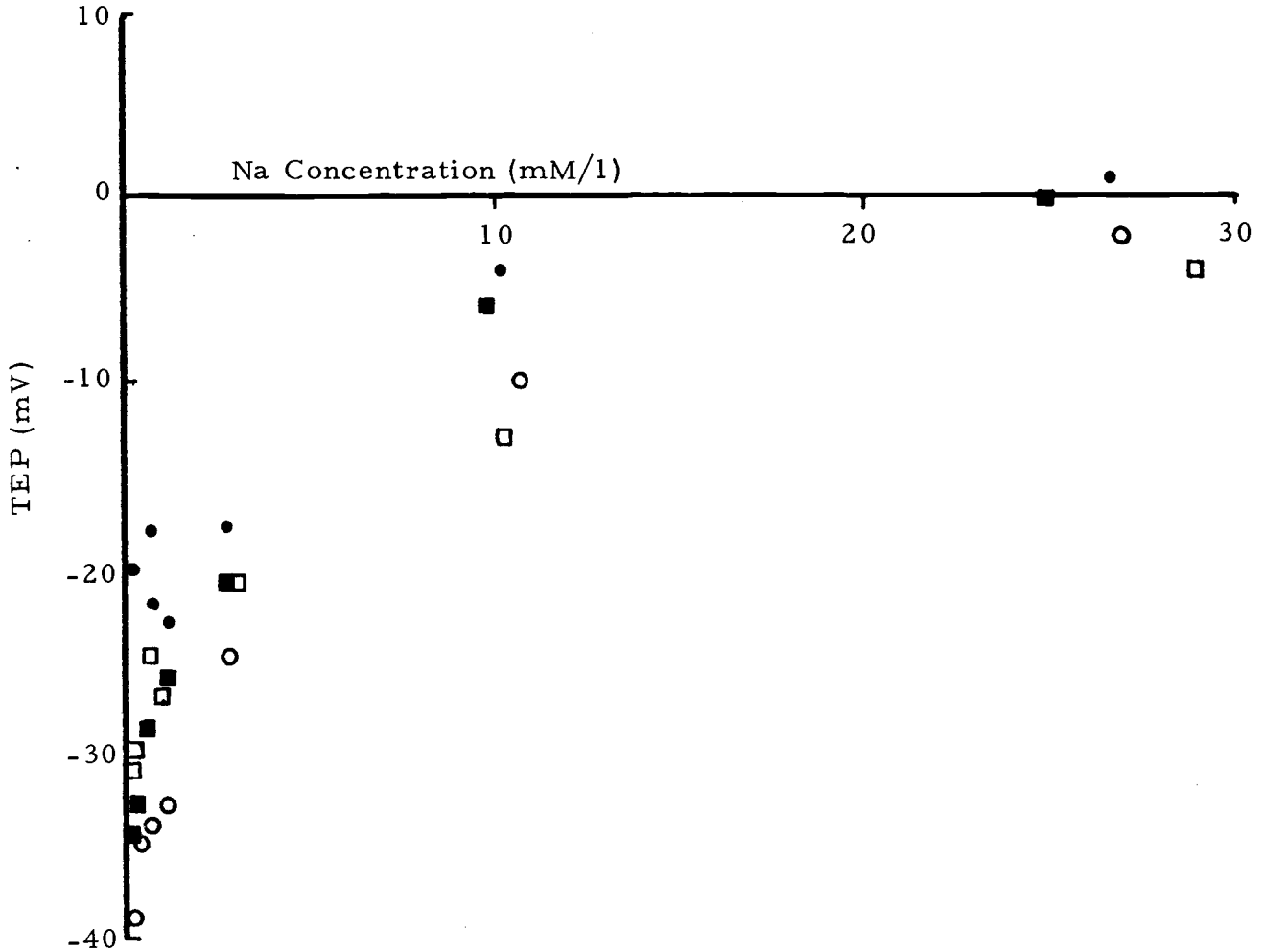

Figure 4. TEP's for two PW acclimated worms exposed to Na_2SO_4 (□ O) or NaCl (■ ●). The sign of the TEP is with respect to the inside of the animal.

adjustment to a change in ion concentration. The TEP is positively correlated with Na concentration and over the range of 0.03 to 30 mM Na/l; it is independent of the anion. The TEP generated by changing the KCl concentration (0.03 to 10 mM/l) gave results similar to Figure 4. This suggested K ions also generate a TEP. The TEP measured on worms in PW were between -30 and -12 mV. Since the worms were not anesthetized, they would frequently move. The contractions caused significant fluctuations in the TEP, for which no correction could be made.

Knowing the electrical gradient and the chemical gradient it is possible to determine if the ion is passively or actively transported. In a steady state the flux ratio (M_i/M_o) is equal to one. If an ion is passively distributed then a calculation of the expected flux ratio (from equation 11) should equal the observed flux ratio. Table 6 lists the calculated and observed flux ratios for worms acclimated to PW. It is evident that the ratios are not equal and both Na and Cl are subject to active transport.

To characterize the Na transport mechanism, experiments were performed measuring the influx of Na over a range of Na concentrations. The basic solution for these studies was PW with different amounts of NaCl added or deleted to give a range of Na concentrations from 0.14 to 4.4 mM/l. Groups of eight worms were acclimated to PW prior to the flux measurements shown in Figure 5.

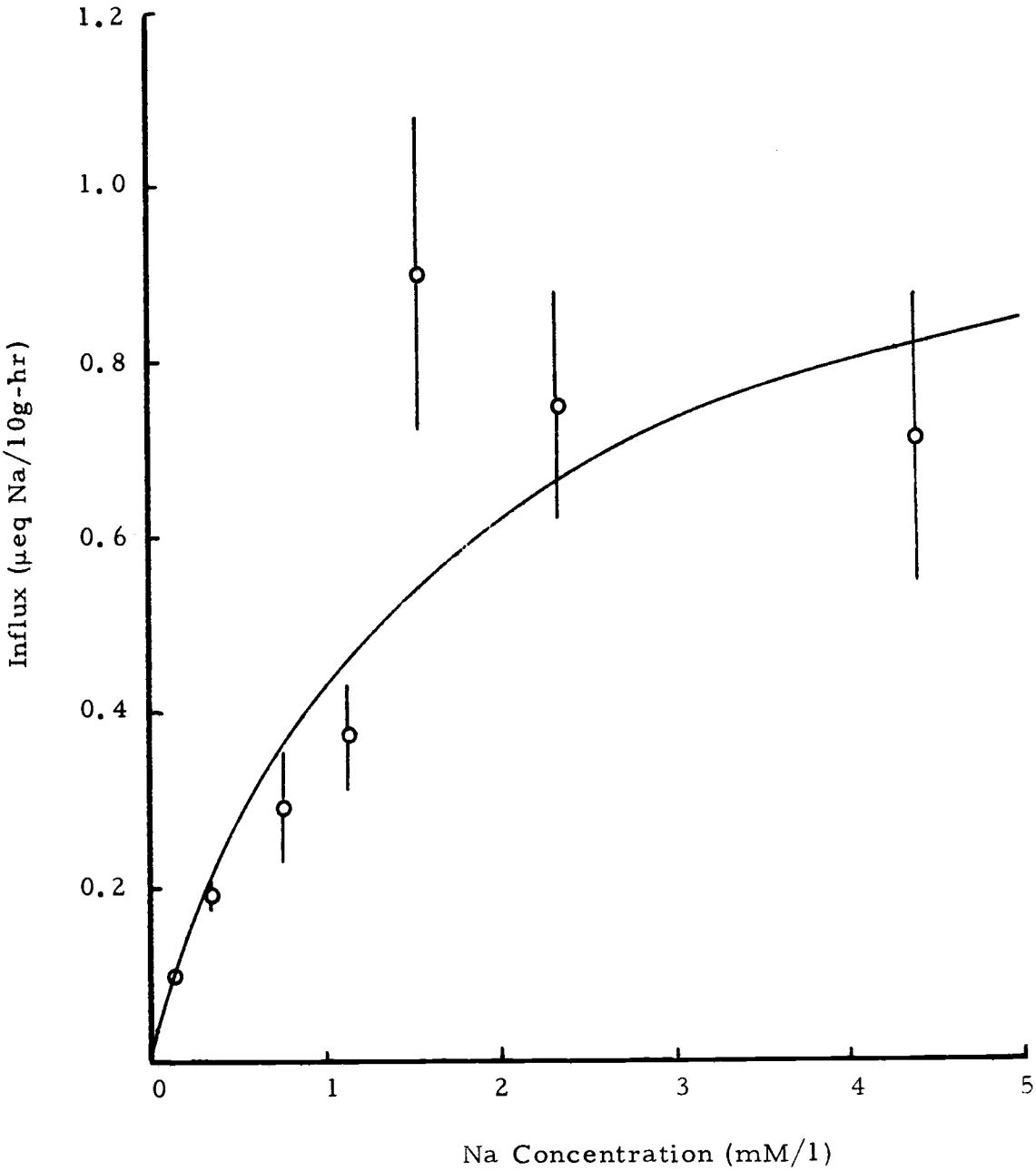

Figure 5. Sodium influx for PW acclimated worms exposed to a range of Na concentrations.

The line was drawn from points selected from a regression analysis of a Lineweaver-Burk plot. The maximum velocity of the transport system, V_m, for PW acclimated worms is 1.12 μeq Na/10 g-hr. The affinity, K_s, of the transport mechanism for Na (i.e., the Na concentration at which the velocity is half V_m) is 1.63 mM Na/l. These data indicate the rate of Na transport is dependent upon the Na concentration at low concentrations and displays saturation kinetics.

Table 6. Calculated and observed flux ratios (equation 11) for PW acclimated worms. The sign of the potential is with respect to the inside of the animal.

Ion	mM/l		Volts	Calc.	Obs.
	C_i	C_o	E	M_i/M_o	M_i/M_o
Na	75	0.67	-0.02	0.004	1
Cl	47	1.25	-0.02	0.059	1

Chloride kinetics have not been determined under similar ionic conditions because of the high Cl concentration in PW, even in the absence of NaCl. However, the mechanism probably behaves in a similar manner.

The kinetic experiments were performed on PW acclimated worms exposed to solutions of different Na concentrations for a short period of time (24 to 36 hours). To determine what influence

prolonged exposure and concentration of the external medium have on the influx, worms were acclimated 10 days to solutions containing elevated Na and Cl concentrations prior to flux measurement. The average M_i for Na and Cl at the different concentrations are shown in Figure 6. The influx for both Na and Cl is a linear function of the respective ion concentration. The slope of the line for Na is five times greater than for Cl (0.25 μeq Na/10 g-hr/mM Na/l compared with 0.049 μeq Cl/10 g-hr/mM Cl/l). Only at 3 mM are Na and Cl transported at the same rate, suggesting the two ions may be transported independently under most conditions.

Control of Ion Transport

Experiments described above (cf Figures 2 and 3) suggested the presence of delicately controlled ion regulatory systems. These systems maintain the CF ion composition constant whether the animals are in PW or soil. Some of the details of the mechanisms involved in ion regulation are accessible to study while the animals are returning to a steady state. When worms are transferred from soil to PW they initially are not in a steady state but return within seven days. Several studies were performed to determine the mechanisms involved in re-establishing a Na and Cl steady state. Groups of eight worms were transferred from soil to PW for various lengths of time (0 to 95 hours) before they were separated

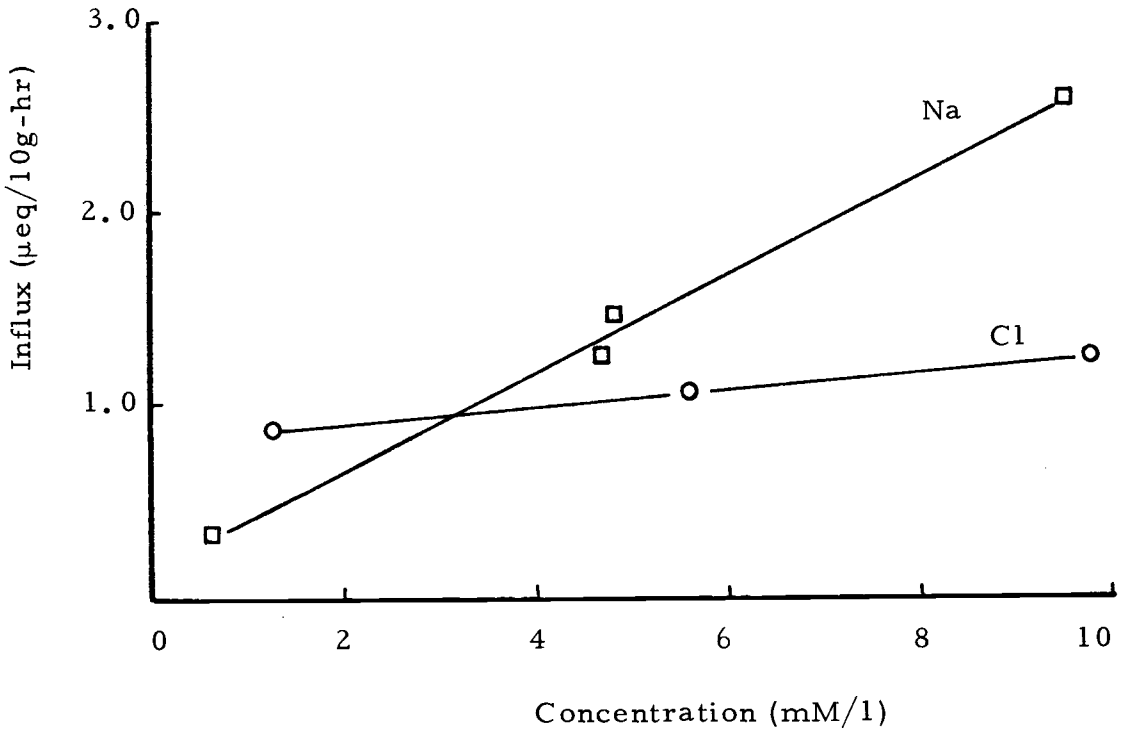

Figure 6. Sodium and chloride influxes for worms acclimated to elevated Na and Cl concentrations.

into individual containers for flux measurement. Three groups of worms were used for both Na and Cl flux studies. The fluxes are expressed as mean values over the interval of each flux experiment and the data for Na and Cl are presented in Tables 7 and 8 respectively.

Table 7. Na fluxes for worms transferred from soil to PW. The values are means for the entire flux experiment.

Hours in PW	N	μeq Na/10 g-hr		
		M_i	M_o	M_n
0-37	8	1.16 ± 0.12	-0.16 ± 0.05	1.00 ± 0.12
38-82	8	0.75 ± 0.06	-0.52 ± 0.05	0.23 ± 0.05
82-130	8	0.52 ± 0.14	-0.51 ± 0.14	0.01 ± 0.02

Table 8. Cl fluxes for worms transferred from soil to PW. The values are means for the entire flux experiment.

Hours in PW	N	μeq Cl/10 g-hr		
		M_i	M_o	M_n
0-30	8	2.61 ± 0.24	-1.17 ± 0.11	1.44 ± 0.15
30-95	8	1.50 ± 0.19	-1.25 ± 0.15	0.25 ± 0.06
95-167	8	0.76 ± 0.06	-0.71 ± 0.06	0.05 ± 0.05

Most of the Na and Cl are taken up during the first interval but some is accumulated in the second interval. By the last interval the animals were in a steady state. The mechanisms employed by earthworms for accumulating Na and Cl differ.

During the first 37 hours in PW, worms increased their Na influx 223 percent and decreased the efflux by 69 percent of the steady state values observed in the third interval. The result was a net influx of 1.0 μeq Na/10 g-hr. During the second interval (38 to 82 hours) the M_o returned to the steady state level but the M_i was 144 percent above the steady state influx. Na was accumulated during this interval at the mean rate of 0.23 μeq Na/10 g-hr.

During the first 30 hours in PW the Cl influx and efflux were higher than the steady state rate observed in the third interval (344 and 165 percent respectively). The net flux was 1.44 μeq Cl/10 g-hr. Between 30 and 95 hours, Cl was accumulated at a lower rate of 0.25 μeq Cl/10 g-hr because M_i was reduced; M_o was not changed appreciably.

For Cl, the steady state fluxes measured in the third interval (95 to 167 hours) are similar to those listed in Table 4 for animals acclimated to PW. However, the Na fluxes between 82 and 130 hours are significantly higher than for acclimated animals. Apparently the Na transport system requires more time than the Cl system to reach the fully acclimated steady state rate. Both transport systems,

however, are changeable to compensate for variations in the worms body fluids caused by changes in the external environment.

When worms enter PW there is both an increase in water content and a dilution of the body fluids. It is important to know which of these changes controls the ion regulatory mechanisms. By exposing PW acclimated earthworms to distilled water (DW), it is possible to reduce the CF ion concentration (effectively a CF dilution) without changing the total water content (88 percent). Lumbricus will not tolerate prolonged exposure to DW (approximately 50 percent mortality in five to seven days). Although populations of worms differ quantitatively in their response to DW, they all behave qualitatively alike. One group of seven worms exposed to DW for three days had CF concentrations of 70 $\pm$ 2 mM Na/l, 3 $\pm$ 0 mM K/l and 33 $\pm$ 3 mM Cl/l. PW animals from the same population had rather high CF concentrations of 84 $\pm$ 2 mM Na/l, 3 $\pm$ 0 mM K/l and 54 $\pm$ 2 mM Cl/l. This is a reduction of the Na and Cl concentrations of 17 and 39 percent respectively. Assuming the extracellular space is equal to the Cl space, 3.7 ml, the Na loss would amount of 52 μeq/10 g and Cl of 70 μeq/10 g.

The rate of ion loss can be determined by measuring the appearance of ions in a distilled water bath. Transferring worms from PW to DW results in an initially high M_o for the first four hours, ranging from -0.35 to -2.39 μeq Na/10 g-hr. This probably

is, in part, due to a handling effect. The efflux between four and 21 hours stabilizes and for 10 animals the mean value was -0.28 ± 0.05 μeq Na/10 g-hr. The efflux of Na to DW has been followed for seven days in an experiment involving eight worms. The animals had about the same rate of loss during the entire experiment. Comparable experiments following the Cl loss have not been performed due to the difficulty in measuring Cl in dilute solutions. The uncontrolled loss of Na and presumable Cl may be a factor in the earthworms inability to withstand extended exposure to distilled water.

Although earthworms exposed to DW do not change their Na efflux significantly, they are able to accelerate their transport systems for both Na and Cl when returned to PW. The result is a recovery of the CF ion deficit incurred during salt depletion.

The fluxes measured for 28 hours on salt depleted animals re-introduced to PW are given in Table 9. Both Na and Cl were taken up in net amounts. Cl was accumulated at a faster rate than Na. For salt-depleted animals the mechanism of accumulation of both Na and Cl is primarily an increase in M_i. The M_o for Na was higher than normal values observed in PW acclimated animals, however, the M_o for Cl was reduced (cf Table 4). These data support the hypothesis that the reduction in CF ion concentration and not a change in water content is the stimulus controling the earthworms

ion transport systems.

Table 9. Mean Na and Cl fluxes for salt-depleted worms re-introduced to PW.

Ion	N	μeq/10 g-hr		
		M_i	M_o	M_n
Na	4	1.78 ± 0.53	-0.67 ± 0.19	1.11 ± 0.51
Cl	4	2.24 ± 0.43	-0.38 ± 0.36	1.86 ± 0.32

Since salt depletion stimulates both Na and Cl transport mechanisms, an experiment was performed to determine if there is a dependency between them. Six worms, salt depleted for four days, were placed in individual containers having either 0.35 mM Na_2SO_4 or 1.30 mM KCl and the Na and Cl flux, respectively, was measured over 20 hours (Table 10). Potassium is not accumulated in net amounts and it is assumed that SO_4 does not penetrate readily so a counter ion is not available, in the bath, to follow an ion transported into the animal. These results are similar to the fluxes presented in Table 9 where the counter ions were present in the solution. Sodium and chloride can be accumulated independently.

To maintain electrical neutrality, an ion exchange system must be involved. The animals are ammonotelic and either NH_4 or H ions could be exchanged for Na; HCO_3 ions could be exchanged

for Cl. These exchange systems remain to be investigated.

Table 10. Mean Na and Cl fluxes for salt depleted worms exposed to 0.3 mM Na_2SO_4 or 1.25 mM KCl respectively.

Ion	N	μeq/10 g-hr		
		M_i	M_o	M_n
Na	3	0.58 ± 0.05	-0.36 ± 0.01	0.22 ± 0.04
Cl	3	1.69 ± 0.60	-0.67 ± 0.32	1.02 ± 0.29

DISCUSSION

Water Balance

Earthworms living in soil or PW have a characteristic water content for each of the two environments which is quite stable after the animals are fully acclimated. When soil acclimated worms enter PW they increase their body weight 15 percent by the net accumulation of water. Several experiments and observations have bearing on the causes of water accumulation and the final stabilization.

The water activity and availability in PW is greater than soil, increasing the driving force for the entry of water. Yet some worms initial rate of urine production is low, and apparently independent of water entry. This is indicated by the low CF clearance value observed for some of the soil acclimated worms (10 μl/10g-hr) as compared with the PW acclimated worms (75 μl/10 g-hr). Consequently, the water content of the worms increases. Within two days the weight reaches a steady state when the uptake and loss become equal.

Several factors may be involved in the weight stabilization. Animals in PW appear to be more "turgid" than when in soil. The development of a turgor pressure may restrict some of the osmotic entry of water. After several days in PW, much of the gut contents are voided and during this time the rectum may begin to expel a

dilute rectal fluid. Thus the rectum may serve as an auxillary excretory organ as proposed by early workers (Maluf, 1939; Wolf, 1940). Finally there may be a reduction in the skin permeability to water. Because of the difficulties in measuring the osmotic uptake of water, this last mechanism has not been evaluated.

Some of the mechanisms employed by earthworms in maintaining a water steady state are similar to those of fresh water animals. Ramsay (1949 a, b) has shown that the presumptive urine entering the nephridia is iso-osmotic with the body fluids but is eliminated as a dilute fluid containing only 55 mOsM total solute. Boroffka's (1965) studies have demonstrated that the dilute urine is formed in the distal part of the nephridium by active reabsorption of Na. The urine remaining hypo-osmotic because of the relative water impermeability of the distal part of the nephridia. My studies of Inulin and Dextran clearance indicate the presumptive urine entering the nephridia is coelomic fluid. Thus the earthworm excretory system appears to be functionally similar to the "filtration"-reabsorption mechanism employed by vertebrates, mollusks and crayfish (Potts and Parry, 1964; Kirschner, 1967).

The rate of urine production has not been measured directly. Bahl (1945, 1947) estimated an excretion rate in Pheretima of 45 percent of its body weight per day. This is probably an overestimate. Most fresh-water animals have an excretion rate of about

one half this value, i.e. 10 to 30 percent of their body weight per day (Shaw, 1963; Kirschner, 1967; Prosser and Brown, 1962). The CF clearance measurements in this study indicate the maximum rate of nephridial excretion by earthworms is 75 μl/10 g-hr or 18 percent of their body weight per day. Since some water is probably reabsorbed by the nephridia, this value is higher than the nephridial excretion rate.

Most fresh-water animals do not use their gut in hyper-osmotic regulation (Shaw, 1963). Any drinking of fresh water usually adds to the animals water problem and does not contribute significantly to their ion balance (Frank and Allee, 1950). Earthworms are similar to other fresh-water animals in that drinking is minimal. However, earthworms apparently do use their gut in some aspects of their water balance, at least while living in fresh water.

Analyses of the rectal fluid indicate PW acclimated worms elaborate a dilute fluid similar in composition to the nephridial fluid (Ramsay, 1949 a, b). Wolf (1940) has estimated the rate of excretion of rectal fluid at 22 μl/10 g-hr. This rate of excretion could make up for some of the water reabsorbed from the nephridia and put the total fluid volume excreted near 75 μl/10 g-hr. In a steady state, this would equal the water uptake rate and is considerably lower than the 250 μl/10 g-hr estimate by Wolf (1940).

The production of a rectal fluid hypo-osmotic to the body fluids

has also been reported for some insects, both terrestrial and aquatic (Phillips, 1964, a, b, c; Ramsay, 1953). The gut fluid in insects is derived, in part, from the malpighian tubules. This is not the case in Lumbricus terrestris, since all of the nephridia open onto the skin.

The mechanisms involved in the production of the dilute rectal fluid are largely unknown. However, some information is available allowing a tentative hypothesis similar to one proposed by Semal (1959). Blood entering the posterior part of the esophagous at the level of the calciferous glands is filtered into the esophageal lumen. The presence of large blood sinuses separated from the esophageal lumen by a thin layer of cells is structurally suited for filtration (Semal, 1959). This fluid is probably similar to the CF. The "secretory" cells in the epithelium of the calciferous gland reabsorb the chloride from the lumen in exchange for bicarbonate. The bicarbonate is precipitated out of the fluid as $CaCO_3$ (Robertson, 1936). Measurements of the crop fluid, just posterior to the esophagous, largely substantiate this hypothesis (see Table 5). The crop fluid is nearly iso-osmotic with the CF. Kamemoto et al. (1962) has shown that the CF is similar in content and composition to the blood. The Na, K and total solute concentrations of crop fluid are similar to the CF. However, the Cl concentration in the crop is only 2 mM/l compared with 47 mM/l in the CF. Since

fluid in the anterior part of the intestine is not expelled out the mouth, the fluid must proceed posteriorly.

As the fluid is passed down the intestine, Na is reabsorbed. The intestine must be relatively impermeable to water allowing the formation and retention of a dilute fluid in the lumen. There are several reported observations suggesting a mechanism for the reabsorption of Na. Tillinghast (1967) has established that much of the ammonium voided by Lumbricus is via the rectum. Therefore Na may be transported out of the intestine in exchange for NH_4. Silvia and Boettiger (1967) have measured the transepithelial electrical potential across the anterior intestine of Lumbricus. Their observations are consistent with the transport of Na from the intestinal lumen (lumen electrically negative to the serosa).

The rate of intestinal fluid formation has been estimated by Wolf (1940). Inserting a canula into the anterior part of the intestine he observed a fluid production rate of about 22 μl/10 g-hr. A single observation on the rate of rectal fluid excreted was similar, indicating little net water reabsorption.

The interesting feature of the entire earthworm intestinal system is its basic similarity to a vertebrate nephron or the earthworms own nephridium. The filtration-reabsorption system allows the animal to void calcium, some metabolic wastes (CO_2 and NH_4) and water but with a minimum loss of Na and Cl. Some of the

mechanisms used are quite similar to those employed in the animals over-all ion regulation discussed in the next section.

Ion Balance

Fresh water animals are hyper-regulators in dilute salt solutions. The CF osmolarity of Lumbricus acclimated to PW is about 160 mOsm/l which is in good agreement with the estimate by Ramsay (1949a). Sodium and chloride comprise the majority of the total solute at 75 and 47 mM/l respectively which are similar to values obtained by Kamemoto et al. (1962). The potassium concentrations in the CF is low (3 mM/l) and Kamemoto et al. (1962) have estimated the CF calcium concentration at 5 mM/l; the remaining solutes are unknown but are probably organic (Krishnamoorthi and Krishnaswamy, 1965).

When entering media of high salt concentrations few fresh-water animals are able to regulate their body fluids at the same level. Notable exceptions are the eel and salmon (Smith, 1932; Potts and Parry, 1964; Prosser and Brown, 1962). Usually, fresh-water animals gain salt and/or lose water until they become iso-osmotic or slightly hyper-osmotic to their environment and succumb when their salinity tolerance is exceeded (Smith, 1932). Lumbricus behaves like most fresh-water animals (Stephenson, 1945; Ramsay, 1949a). Although earthworms are not able to maintain their body

fluids hypo-osmotic to their environment, they are capable of ion regulation in more concentrated media. Figure 1 illustrates that the CF sodium concentration becomes equal to the medium at 126 mM/l but the difference between Na and Cl remains relatively constant over the entire range. Ramsay (1949a) has shown that CF chloride is maintained below a bath concentration of 220 mM/l. Ramsay (1949a) suggested that this chloride regulation may not be due to active transport. For the CF chloride to be in passive equilibrium with an environmental Cl concentration of 126 mM/l, equation 11 predicts a TEP of 13 mV with the CF negative. For Na to be in passive equilibrium, the TEP would have to be 0 mV. TEP's have not been measured on worms acclimated to higher NaCl solutions so it is not known whether Na or Cl are actively transported or the direction of transport.

Most fresh-water animals (fish, amphibians, crayfish) tolerate exposure to distilled water (Krogh, 1939; Potts and Parry, 1964; Alvarado, 1962). When transferred to DW, these animals initially lose Na and Cl but if the ions are allowed to accumulate in the bath the animals will again re-establish a steady state at reduced bath concentrations (Morris and Bull, 1968; Potts and Parry, 1964). For the crayfish, this "minimum equilibrium" level is less than 0.04 mM NaCl/l (Shaw, 1964). The lower limit for an animal is a function of the rate of loss and the ability to transport ions from

dilute solutions.

When Lumbricus is transferred to DW and the ionic concentration is allowed to increase, they do not reach a steady state within seven days. There are several factors bearing on worms lack of ion regulating capability in DW. When placed in DW the salts lost are primarily Na and Cl in equal amounts. The bath concentration increases to 0.2 to 0.6 mM/l with no indication they are approaching a steady state. Table 1 attests to their inability to ion regulate in dilute NaCl solutions. In part, this may be due to their unchanged rate of Na loss and a low influx. Calcium is necessary for maintaining membrane permeability in cells (Davson, 1964) and may be important for epithelia also. Morris and Bull (1968) have shown that the ammocoetes larvae requires a certain ion balance in the environment; the presence of Ca significantly reduces the rate of Na loss to a Na free solution.

Although earthworms placed in DW cannot re-establish a steady state at very low ionic concentrations, salt depleted worms returned to PW can. While studying the fluxes of salt depleted worms returned to PW, several animals were observed to reduce the bath Na and Cl concentrations below 0.1 mM/l. Potassium was lost but changes in Ca were not monitored. PW initially contains 0.4 mM Ca/l. This may be responsible for the worms ability to take up ions and reduce the bath concentration to low levels, however the precise role of Ca

is not known. When salt depleted worms are returned to PW the rate of Na loss is not changed, however, the Cl efflux is slightly reduced. The mechanism employed by salt depleted worms net uptake of Na and Cl is primarily an increase in the influx (compare Tables 4 and 9).

The uptake of Na and Cl from PW is by active transport. The passive component of the influx can be calculated using equation 11. For Na, the passive influx from PW is only 0.4 percent and for Cl, six percent of the total influx (Table 6). These data are in good agreement with respect to the passive component in other fresh-water animals (Shaw, 1963; Jørgensen and Dales, 1957; Potts and Parry, 1964).

In studying the kinetics of sodium transport, two parameters are generated which allow quantitative comparisons between different animals. They are the maximum velocity of the transport system, V_m, and the affinity of the transport system for an ion, K_s. The rate of Na transport is lower in Lumbricus terrestris than other aquatic animals, with a V_m of about 1 μeq/10 g-hr. Marine crustacea and polychaetes have the highest V_m with rates between 100 and 200 μeq/10 g-hr for both Na and Cl (Potts and Parry, 1964; Jørgensen and Dales, 1957; Fretter, 1955). Fresh-water animals have a transport system of lower capacity than marine animals but higher than earthworms. The V_m for crayfish and salamander is

about 3 μeq Na/10 g-hr (Shaw, 1959; Alvarado, unpublished observations).

The affinity of the Na transport system in Lumbricus is 1.6 mM Na/l which is higher than fresh-water animals but lower than marine. The marine crab, Carcinus, has a K_s of 20 mM Na/l where as the fresh-water crayfish, Astacus, has a K_s of 0.2 to 0.3 mM Na/l (Potts and Parry, 1964).

The earthworms capacity for Na transport, as reflected by V_m, may be limited by the rate of penetration of Na, the turnover time of the transport system and/or the number of transport sites available. The high K_s observed for Lumbricus suggests that a relatively high concentration gradient is necessary to half saturate the transport system. Together the V_m and K_s suggest that the earthworms permeability to Na is rather low.

Few kinetic studies of the Cl transport system in fresh-water animals have been performed. However, unpublished observations by Alvarado on the salamander indicate the Cl and Na transport systems are quantitatively similar. The study relating the effects of acclimation to higher bath ion concentrations to influx indicates that Cl may penetrate the skin faster than Na at low concentrations (< 3 mM) but slower at higher concentrations (see Figure 6). The significance of the differences between Na and Cl transport rates at the different ion concentrations are unknown.

Ussing and Zerahn (1951) were the first to describe the mechanism of ion transport across animal epithelia. Using the isolated frog skin they were able to show that Na was actively transported from the epidermal surface to the corial surface. To maintain electrical neutrality Cl follows passively. This model has been useful in its application to other epithelia (Davson, 1964). However, Krogh (1937, 1939) observed that intact frogs could take up Cl from dilute solutions of KCl without the concomitant uptake of the cation. Jørgensen et al. (1954) demonstrated that frogs actively transported Na and Cl independently.

An exchange mechanism has been postulated for the independent transport of Na and Cl. Either NH_4 and/or H ions are exchanged for Na and HCO_3 is exchanged for Cl. The presence of exchange mechanisms in epithelia of fish, frogs, salamanders and crayfish has abundant support (Maetz and García Romeu, 1964; Krogh, 1937, 1939; Jørgensen et al., 1954; Shaw, 1960; Dietz et al., 1967).

Salt-depleted earthworms are able to take up net amounts of Na and Cl when placed in Na_2SO_4 and KCl respectively. The counter ions in each case are considered to have low penetrability (Table 10). To maintain electrical neutrality these worms would also have to have an exchange mechanism in their skin. The presence of ion exchange mechanisms in the intestinal tract has been suggested above.

Mechanisms controlling ionic and osmotic regulation are apparent during the course of earthworms acclimation to PW. The stimulus appears to be the dilution of the coelomic fluid. The response is an accelerated influx. It is not known whether the transport sites are turned over at a faster rate or whether there is an increase in the number of sites. The efflux of Na is not changed, however, Cl efflux may be reduced. The receptors responsible for the control of earthworms hydromineral metabolism are largely unknown. Kamemoto (1964) has implicated a brain factor involved in earthworms water and salt balance. Experiments involving brain extirpation from soil and PW acclimated animals have been inconclusive. Wherever the receptors are located, they are sensitive to changes in the CF and initiate changes, rapidly, which return the CF to normal ionic concentrations.

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