AN ABSTRACT OF THE THESIS OF

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A primary feature of osmoregulation in fresh water vertebrates is the delicate balance between osmotic uptake of water and renal excretion of water. Changes in osmotic load lead to alterations in renal function which preserve this balance. Little is known of the mechanisms of these alterations or their time courses. The primary objective of this investigation was to analyze the effect of abrupt changes in osmotic load on renal function in larval Ambystoma gracile and Dicamptodon ensatus. A second objective was to compare renal parameters as measured in unanesthetized, intact salamanders with available data obtained by a variety of complex surgical techniques.

No significant differences were found between the clearances of ^{14}COOH-inulin and unlabeled inulin measured simultaneously. The mean clearance of ^{125}I-sodium iothalamate (glofil-^{125}I) was slightly grater than mean inulin clearance although the difference was not significant. ^{14}COOH-inulin was used to measure glomerular filtration rate (GFR) in all subsequent experiments.

GFR was calculated from the rate of excretion of inulin to the bath. A seasonal effect was noted. The mean GFR (in ml/10g-hr) of A. gracile measured during winter was 0.056 ± 0.016 (5). In spring the GFR was 0.124 ± 0.012 (5) and in summer-fall it was 0.107 ± 0.017 (10). Summer-fall D. ensatus had a GFR of 0.166 ± 0.019 (19).

GFR can be adjusted in relation to the osmotic load facing the animal. The pooled mean GFR for A. gracile was 0.098 ± 0.011 (20) in tap water; 0.077 ± 0.010 (15) in 100 mOsm (adjusted with sucrose); 0.066 ± 0.008 (20) in 200 mOsm; and 0.018 ± 0.004 (15) in 300 mOsm. For D. ensatus (summer-fall) the GFR was 0.166 ± 0.019 (9) in tap water; 0.113 ± 0.016 (9) in 100 mOsm; 0.030 ± 0.010 (9) in 200 mOsm; and 0.018 ± 0.006 (9) in 300 mOsm. The latency in reduction in GFR when the bath concentration was abruptly elevated to 200 mOsm was two to three hours. The latency for an increase in GFR when animals in 200 mOsm were returned to tap water was approximately five hours. Changes in GFR probably reflect glomerular intermittency as the tubular maximum for glucose decreased from 0.13mg/10g-hr to 0.01 mg/10g-hr while GFR decreased from 0.048 ml/10g-hr to 0.002 ml/10g-hr in D. ensatus.

Renal tubular function was assessed in spring A. gracile and winter D. ensatus by collecting urine in rubber bags. Urine production was constant for the duration of the experiment (12 hours) and no water was reabsorbed across the skin from the urine in the bags.

Urine production rate ($\dot{V}$ in ml/10g-hr) for A. gracile in tap water was 0.077 ± 0.007 (19). This decreased to 0.019 ± 0.002 (16) in 200 mOsm. GFR (ml/10g-hr) decreased from 0.102 ± 0.007 (19) to 0.031 ± 0.004 (16) under the same conditions. Inulin U/P values increased from 1.4 in tap water to 1.7 in 200 mOsm animals ($P < 0.05$). In tap water $\dot{V}$ was 0.040 ± 0.002 (4) for D. ensatus. This decreased to 0.003 ± 0.001 (4) in 200 mOsm. Under the same conditions GFR decreased from 0.056 ± 0.004 (4) to 0.006 ± 0.000 (4) and inulin U/P values increased from 1.4 to 2.0 ($P < 0.05$).

In A. gracile urine total solute (mOsm/l) was 36 ± 2 (19) in tap water and 86 ± 8 (14) in 200 mOsm sucrose. Urine ion concentrations (μeq/ml) in tap water were: Na^+, 10.4 ± 0.7 (19); K^+, 3.7 ± 0.4 (19); Cl^-, 6.6 ± 0.4 (19). In 200 mOsm sucrose these were: Na^+, 26.9 ± 3.7 (15); K^+, 6.2 ± 0.9 (15); Cl^-, 17.3 ± 2.2 (15). Urine phosphate concentration in tap water was 2.69 ± 0.22 (14) μAtm P/ml. Urinary concentrations of nitrogenous waste products (μAtm N/ml) in A. gracile in tap water were: urea, 1.68 ± 0.29 (13); ammonia, 2.21 ± 0.33 (10).

Renal tubular handling of several solutes was assessed in spring A. gracile. Fractional reabsorption of sodium, potassium and cloride were 92, 71 and 94 percent respectively in tap water. These values decreased to 84, 57 and 86 percent respectively in 200 mOsm. Fractional reabsorption of urea was about the same in both groups (40% in tap water; 36% in 200 mOsm). There was a slight net

secretion of ammonia into the nephric tubules of both groups. Phosphate was reabsorbed in the tubules of some animals and concentrated in those of others.

Glomerulotubular balance for sodium and chloride was indicated by a linear decrease in the rates of absolute reabsorption of these ions as GFR decreased.

Na^+, K^+-ATPase was assayed in kidney tissues taken from D. ensatus adapted to tap water and 200 mOsm sucrose (2 weeks). Enzyme activity in tap water adapted animals was 20.0 ± 6.1 (7) μmoles Pi/mg protein-hr; in 200 mOsm adapted animals this was 8.3 ± 2.0 (7) μmoles Pi/mg protein-hr.

Renal Function in Urodele Amphibians

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RENAL FUNCTION IN URODELE AMPHIBIANS

INTRODUCTION

Historical Perspective

The currently held concepts of renal function originated with Carl Ludwig who, in 1844, first proposed that urine is produced primarily by the filtration of plasma in the glomeruli followed by reabsorption of water and "usable" solutes from the protein-free filtrate in the nephric tubules (Smith, 1964).

Ludwig, however, was not the only biologist to publish ideas pertaining to renal function. In 1842 an English physician named William Bowman accurately described the nephron and proposed that urine formation was primarily a process of secretion of waste materials from the peritubular capillaries into the tubular lumenae. This theory allowed for glomerular filtration but it suggested that the filtrate functioned to wash the secretions through the nephric tubule and thus played a minor role in the over-all function of the kidney. Perhaps the most remarkable feature of these theories of renal function is that they were both based solely on anatomical studies and deductive reasoning. That Ludwig was basically right, as was Bowman to a degree, is truly amazing. The synthesis of the two theories was long in coming and the polemic between

"filtration" and "secretion" adherents raged into the third decade of this century.

These two theories represented more than merely divergent views within medicine and physiology. Ludwig was an outspoken mechanist, whereas Bowman was a vitalist. For reasons which now are unclear the vitalists found Bowman's interpretation more "vitalistic" and clung tenaciously to his secretion theory.

In 1874 Rudolph Heidenhain reported that when the dye indigo carmine was injected into the venous circulation of the rabbit it appeared first in the cells and lumenae of the nephric tubules and was never seen in the space between the glomerulus and its associated Bowman's capsule or in the capsule itself. He thus contended that it was secreted through the tubular cells from the peritubular capillaries into the lumen and not filtered. As a result of these findings the secretion theory gained a foothold that was not relinquished for some fifty years.

Evidence for the filtration-reabsorption theory accumulated slowly. It was shown by Ribbert (1883, cited by Wearn and Richards, 1924) that careful extirpation of the medulla of the remaining kidney of a heminephrectomized rabbit resulted in a marked diuresis. It was concluded that water is reabsorbed in the medulla of the intact kidney. Heidenhain's experiment was repeated by von Sobieranski (1895, cited by Wearn and Richards, 1924) using larger amounts

of indigo carmine and he found the dye within Bowman's capsule, thus implicating filtration. Finally, Arthur R. Cushney (1902) showed that following the injection of an isosmotic solution of sodium chloride and sodium sulfate into a rabbit to produce saline diuresis, the changes in the urinary concentration of the two salts follow different time courses. The chloride ion in the urine becomes more dilute with time than does sulfate. This experiment was repeated by Yagi and Kuroda (1915) with renal artery constriction (which would slow the perfusion rate of the tubules) and it was found that the difference in the time courses of the urinary concentrations of chloride and sulfate was even greater. The chloride became diluted much faster under these conditions while the sulfate concentration maintained its slow rate of decrease. The conclusion in both experiments was that chloride is reabsorbed to a greater extent than sulfate. Although none of these experiments could prove the filtration-reabsorption theory when taken alone, it was concluded by Cushney, who assembled and evaluated the material in 1917, that this theory best fit the data (Smith, 1964).

The final proof of Ludwig's views came from the work of A. N. Richards and his colleagues who developed the micropuncture technique. Initial studies were done on the frog; later studies were performed on the salamander, Necturus maculosus, which is ideally suited because of the large size and ready accessability of its renal

tubules. This illustrates the importance of the comparative physiology approach in attacking a basic physiological problem. Using a quartz capillary tube which was drawn out to a sharp tip with an inside diameter of 10-20μ and mounted on a micromanipulator, these investigators succeeded in withdrawing samples of fluid from between a glomerulus and its associated Bowman's capsule (Wearn and Richards, 1924). Qualitative tests showed that protein was absent from this fluid, providing the first empirical proof of the filtration theory. Further qualitative tests were made on this fluid and simultaneous samples of bladder urine. It was shown that glucose and chloride are present in large quantities in the filtrate while no glucose and only traces of chloride ion can be seen in bladder urine. This provided proof for the second part of Ludwig's theory, that of reabsorption in the tubules.

Shortly thereafter quantitative methods were devised for chloride concentration determinations and it was shown that there is a considerable drop in the concentration of this ion between the glomerular filtrate and the bladder urine (Wearn and Richards, 1925).

Water and chloride ions were found to be reabsorbed over the entire length of the tubule while the concentration of chloride ion does not decrease until the fluid reaches the distal tubule (Walker et al., 1937). Walker and Hudson (1937b) showed that phosphate is occasionally reabsorbed in the proximal tubule but is usually concentrated

due to reabsorption of water and that it is not secreted. Urea is also not secreted but is concentrated by the reabsorption of water (Walker and Hudson, 1937a). Ammonia is secreted into the distal tubule and is probably manufactured from some precursor in the tubule cells (Walker, 1940). It was shown by Bott (1962) that sodium, like chloride, is reabsorbed in an isosmotic solution in the proximal tubule but becomes increasingly more dilute in the distal tubule. These are a few of the many studies performed on Necturus tubules which, by the late 1950's, fixed the site of tubular transfer of water and many solutes.

Although Richards and his colleagues had convincingly demonstrated that filtration and reabsorption were the primary processes in urine formation, their data did not rule out the existence of secretion.

At about the same time that Wearn and Richards were performing the first micropuncture experiment, another group was engaged in whole kidney studies. Eli K. Marshall and his co-workers showed that when plasma urea concentration is increased in a dog, the urinary excretion of this product increases proportionally. On the other hand, increasing the plasma concentration of the dye, phenol red, results in increased excretion only up to a critical plasma level at which point the excretion of the dye will increase no further, even when the plasma concentration is further elevated. The

interpretation was that the urea is filtered but the phenol red has to be transferred into the tubular lumen by some process that is rate limited and thus becomes saturated. It was shown a short time later that both urea and phenol red behave this way in the frog (Marshall and Crane, 1924). This is considered to be among the first convincing evidence of tubular secretion (Smith, 1964).

Two years later Marshall and Grafflin (1928) described the structure and function of the kidneys of the marine fish, Lophius piscatorius. The peculiarity of this kidney is that it has only a very few vascular structures which resemble glomeruli (about one for each 2,000 tubules). The urine of these animals is more concentrated than the plasma with respect to several solutes, including Mg^{++}, Ca^{++}, $SO_4^{=}$ and creatinine. This provided conclusive evidence for renal tubular secretion.

Of course the old controversy was not settled immediately but over the years it has been recognized that the definitive urine is formed by three processes: the hydrostatically driven formation of a protein-free filtrate in the glomeruli; the active or passive transfer of materials from the tubule lumenae back to the blood, a process called reabsorption; and the active secretion of waste products into the tubule lumenae. This last process can occur in either of two ways; substances can be transferred across the tubule wall from the peritubular capillaries or they can be formed from precursers

in the tubule cells themselves and then be secreted into the lumen.

In recent years techniques such as micropuncture have been refined and applied to other animals. New ultra-micro chemical analyses have been devised. The use of radioactive isotopes which allows the detection of minute quantities of the substance in question has become widespread. The use of isotopes allows the separation and evaluation of unidirectional fluxes across tubular epithelia. The development of microelectrodes (Ling and Gerard, 1949) permitted the measurement of electrical gradients which is critical to the interpretation of ionic fluxes.

In 1958 a group headed by A. K. Solomon combined many of these techniques and applied them in an intensive study of tubular function in Necturus maculosus. This method is referred to as stopped flow micropuncture (Shipp et al., 1958). With this procedure a small volume of perfusate is held stationary in a nephric tubule for a certain length of time. During this period unidirectional fluxes of water and specific solutes are measured using radioactive tracers and chemical analyses. With microelectrodes, electrical potential gradients between the lumen and the outside of the tubule were measured. Using these techniques on the proximal tubules it was shown that sodium is reabsorbed against a chemical and an electrical gradient (Whittembury and Windhager, 1961). This reabsorption is energy dependent and is inhibited by ouabain, an

inhibitor of active transport of sodium (Schatzmann, Windhager and Solomon, 1958). The movement of water out of the proximal tubule is entirely dependent upon the active transport of sodium (Windhager et al., 1959; Whittembury et al., 1959). Chloride ion is reabsorbed passively following the electrical gradient set up by sodium transport (Giebisch and Windhager, 1963). Potassium ion is not reabsorbed in the proximal tubule (Oken and Solomon, 1963; Solomon, 1963).

Having learned a great deal about the physiology of the Necturus proximal tubule, Solomon and his group next turned to the distal tubule. Maude, Shehadeh and Solomon (1966) demonstrated that both sodium and potassium are transported out of the distal lumen in a hyperosmotic solution, leaving the filtrate diluted with respect to sodium. Sodium and potassium are also actively reabsorbed in the distal tubules of Amphiuma, another urodele. Potassium can be actively secreted when Amphiuma become potassium loaded (Wiederholt et al., 1971).

Micropuncture techniques have also been applied to mammals dating from the work of Walker et al. (1941). Many of the mechanisms first elucidated in Necturus are very similar to those in mammalian tubules although there are also significant differences. This work has been extensively reviewed elsewhere (Giebisch and Windhager, 1964; Pitts, 1968).

The micropuncture experiments revealed much of the intimate

detail of renal function. However, they did not describe the functions of the intact kidney with respect to the osmotic needs of the animal.

A second line of investigation dealt with the function of the intact kidney. It centered around the concept of renal clearance originated by Van Slyke and his colleagues (Austin, Stillman and Van Slyke, 1921). The potential of the clearance technique was first recognized by Homer W. Smith (Smith, 1943b). Renal clearance is defined as the volume of blood (or plasma) cleared of a particular substance during a given interval of time and expressed as the product of the urinary concentration of that substance (U) and the rate of urine formation ($\dot{V}$), divided by the plasma concentration (P) of the substance (i.e. $C = U\dot{V}/P$). It had been realized by Rehburg (1926) that the clearance of a substance which was filtered, but neither reabsorbed nor secreted, would be equal to the filtration rate. Rehburg noted that creatinine was concentrated to a greater extent than any other urinary component and suggested its use as an indicator of glomerular filtration rate (GFR). Smith, however, pointed out the observation of Marshall and Grafflin (1928) that creatinine was secreted in the kidneys of other animals. An investigation of a series of carbohydrates was subsequently carried out by Smith and his colleagues to find a suitable GFR marker. The choice of this group of compounds was based on the premise that mechanisms for the secretion of such nutritionally valuable molecules

probably never arose and that a carbohydrate might be found which was metabolically inert and not reabsorbed by the kidney tubules. After a false start with xylose (Jolliffe, Shannon and Smith, 1932) which was later shown to be partially reabsorbed, it was discovered that such carbohydrates as sorbitol, mannitol and inulin are cleared at precisely the same rate in man (Smith, 1943a). It was further shown that phlorizin, which inhibits the reabsorption of such carbohydrates as glucose and xylose, has no effect on the clearance of inulin (Shannon, 1935). Because the very large size of inulin lowers its probability of passive reabsorption, this compound became the standard marker for the measurement of GFR. Recent attempts to find alternate markers of GFR (sodium iothalamate, vitamin B^{12}, complexes of divalent cations and ethylenediaminetetraacetate, etc.) have met with some success, but inulin remains the standard against which each of these is tested.

The importance of a technique to measure GFR cannot be overestimated. It allows the estimation of the quantity of any given substance filtered (GFR x plasma concentration of the substance). With this datum and the knowledge of the amount excreted ($\ddot{V}$ x urine concentration of the substance) it became possible to determine the role of the tubules in modifying the glomerular filtrate. Thus, if more of the substance is filtered than excreted, some of it must have been reabsorbed. Conversely, if more is excreted than

filtered, it must have been added to the tubular fluid subsequent to filtration, presumably by secretion.

The primary advantage of the clearance technique is that it permits the investigation of the handling of any substance by the tubules without the need for drastic surgical techniques. While the utility of this in clinical applications is obvious, it is also of great importance to basic renal investigation. The ability to measure these tubular phenomena in intact laboratory animals has provided much of the information we now have on the function of the kidneys in mammals. Unfortunately, with the exception of a few studies on anurans, these techniques have not been extensively applied to amphibians. This is particularly true of the urodeles, a group which has been the subject of a massive investigation at the level of the individual tubule.

Another approach to the analysis of renal function has been to evaluate the role of the kidney in the maintenance of homeostasis in the whole animal. The primary focus here has been on how the various processes taking place in the kidney are integrated and controlled with respect to other organ systems to produce adaptive responses in a variable environment.

The Present Study

As indicated above, much of our knowledge of renal physiology

has been obtained from studies on the urodelian kidney at the level of the single tubule. The primary objective has been to elucidate basic mechanisms at this level with relatively little regard for the overall role of the kidney as the major homeostatic organ in the body. The success and importance of these studies is well documented. Given this body of information it would appear advantageous to examine some of the basic renal parameters in intact, conscious, free-swimming animals and to examine the range of capabilities of the various parameters. Larval urodeles are particularly well suited for studies of this type for several reasons:

1. They normally inhabit fresh water.
2. Their skin displays a relatively high permeability to water so that under normal conditions their "osmotic load" is high (Krogh, 1939).
3. The rate of urine production ($\dot{V}$) is proportional to the osmotic load.
4. The urine is dilute in solute indicating extensive alteration of the urine as it passes through the tubule.
5. They do not drink.
6. They do well under laboratory conditions being considerably less sensitive to "handling stress" than many fresh-water animals.

The animals chosen for investigation are larvae of two species

of the family Ambystomatidae, Ambystoma gracile (Baird) and Dicamptodon ensatus (Eschscholtz). The use of these two species offered two advantages. Because they are closely related, their responses to experimental treatments should be very similar, and yet they might be expected to show subtle differences in renal function due to the differences in their respective habitats. Ambystoma gracile generally inhabits ponds or slow-moving streams where the temperature and solute concentration tend to be relatively high. Dicamptodon ensatus generally inhabits small, fast-moving streams where temperature and solute concentration tend to be quite low. The second advantage in the use of these species is that one or the other of these animals is available throughout the entire year.

The objectives of the present study were as follows:

1. To measure GFR, $\dot{V}$ and renal handling of water and various solutes in larval A. gracile and D. ensatus in conditions as close to "normal" as possible (i.e. free-swimming, unanesthetized animals in tap water).
2. To study the changes in renal function when osmotic load is abruptly changed.
3. To study the time course of changes in renal function in response to abrupt changes in osmotic load.

A few studies were done on animals chronically exposed to low osmotic load.

MATERIALS AND METHODS

Collection and Maintenance of Animals

Larval Ambystoma gracile were collected in minnow traps from Owl Creek, east of Corvallis, in Linn County, Oregon. Larval and neotene Dicamptodon ensatus were collected by hand from Parker Creek, located on Mary's Peak in Benton County, Oregon. Additional neotenes of this species were taken by hand from a small tributary of the Nestucca River north of Pacific City, Oregon.

All animals were maintained at 15 ± 1°C in dechlorinated tap water. The water was either flowing through the containers or was changed frequently. All animals were allowed to adjust to laboratory conditions for at least one week and usually longer before being used in experiments. The animals were not fed except in a few cases of long term storage when they were fed small, live salamanders or raw beef heart. Animals were never fed within two weeks of their use in experiments.

Anesthetic

When the use of anesthesia was required the animals were immersed in 0.1 percent tricane methane sulfonate (Finquel, Ayerst Laboratories). The pH of the solution was adjusted to approximate

neutrality with $NaHCO_3$ (1g/1). Anesthesia was complete after 5 minutes for D. ensatus and 20 minutes for A. gracile. Recovery from anesthsia usually occurred between 40 and 60 minutes after transfer to tap water.

Counting of Radioactivity

Carbon-14 was counted with Nuclear Chicago ambient temperature liquid scintillation spectrometer. A toluene-based counting solution developed by Turner (1968) was used (Triton X-100 as a solubilizer, PPO as a primary fluor and POPOP as a secondary fluor). Two ml samples were added to 15 ml of counting solution. Quenching was estimated and corrected by the channels ratio method (Wang and Willis, 1965). Quench standards were prepared with known activities of ^{14}C-toluene standard (New England Nuclear).

Samples containing ^{22}Na were also counted by liquid scintillation spectrometry. In this case 1 ml samples were prepared with 10 ml of counting solution. Quenching was monitered with the channels ratio method but as it was constant no correction was necessary.

Samples containing ^{125}I were plated on aluminum planchets and counted with a Geiger-Mueller gas flow detector (Nuclear Chicago).

In one group of experiments the radioactivity in the bath

containing animals previously injected with ^{22}Na was monitered continuously with a sodium iodide crystal well detector. This was accomplished by pumping the water through a coil located in the well of the crystal. This detector was used in conjunction with a Nuclear Chicago rate meter connected to a 10 mv recorder.

Chemical Analyses

All urine and plasma samples which could not be analysed immediately were stored at -10°C. This had no effect on the various substances assayed, with the possible exceptions of urea and ammonia. These were assayed immediately after sampling. Urea was measured colorimetrically using the diacetylmonoxime-thiosemicarbazide method of Coulombe and Favreau (1963). Ammonia was determined by the Nessler's reaction.

Sodium and potassium concentrations were estimated by flame photometry (precision = ± 1%) after appropriate dilution of the samples. Chloride ion was titrated electrometrically with a Cotlove-type chloridometer (precision = ± 1%) following appropriate dilution. Undiluted urine and plasma samples were analysed for total solute concentration with a Mechrolab vapor pressure osmometer (precision = ± 2%).

Phosphate ion concentration was determined colorimetrically using the method of Murphy and Riley (1962). Plasma samples were

deproteinized with 10 percent trichloroacetic acid (TCA) prior to analysis.

The chemical determination of inulin was performed using the modification of Nakamura (1968) of the method of Roe et al. (1949).

Glucose was determined enzymatically with the Glucostat kit (Worthington Biochemical). Plasma samples were deproteinized with a 1:1 mixture of 2% $ZnSO_4$ and 1.8% $Ba(OH)_2 \cdot 8H_2O$ (pH = 7).

Detailed protocols of chemical analyses are given in the Appendix.

Measurement of Inulin Space

The volume of distribution of inulin (inulin space) was measured using a $^{14}COOH$ labeled inulin compound (New England Nuclear) by a method similar to that employed by Walker (1971). A known quantity of inulin was injected subcutaneously into each of several anesthetized animals. Each animal was then placed in 100 ml of tap water. At subsequent intervals small groups of the animals were sacrificed and plasma inulin concentration was determined. From the knowledge of the amount of inulin injected, the amount excreted and its concentration in the plasma, the inulin space can be estimated from:

$$S = \frac{T - E}{P} \qquad (1)$$

Where: S = Inulin Space (ml)

T = Total inulin injected (DPM)

E = Inulin excreted (DPM)

P = Plasma inulin concentration (DPM/ml)

Measurement of Glomerular Filtration Rate (GFR)

GFR can be measured as the renal clearance of inulin:

$$GFR = \frac{U}{P} \dot{V} \qquad (2)$$

Where: GFR = ml/hr

U = Urine inulin concentration (DPM or μg/ml)

P = Plasma inulin concentration (DPM or μg/ml)

$\dot{V}$ = Rate of urine production (ml/hr)

This relationship can be simplified for aquatic animals as follows:

$$GFR = Q/P \qquad (3)$$

Where: GFR = ml/hr

Q = Rate of appearance of inulin in the bath (DPM or μg/hr)

P = Inulin concentration in the plasma (DPM or μg/ml)

The use of equation (3) relies on the following assumptions: the kidneys must be the only route of loss of the marker; the animal must not drink the bathing medium; the urine must not be altered (by storage in the bladder) before it enters the bath; and the compound must be stable in the bath. Several experiments were performed to test these assumptions.

To determine the extent of extra-renal loss of ^{14}COOH-inulin or ^{125}I-sodium iothalamate (Glofil I-125; Abbott Laboratories) each of several salamanders (A. gracile), previously injected with the appropriate marker (1 μCi), was anesthetized and its cloaca blocked with a "purse-string" ligature. Each animal was then placed in 100 ml of tap water and the radioactivity of the bath was monitored by taking 1 or 2 ml samples at 3 hour intervals during the following 6 hours.

Another experiment was performed to determine the extent to which the salamanders drink water. Several D. ensatus were placed in individual containers in 25 ml of tap water containing approximately 10^6 CPM of ^{14}COOH-inulin. After 24 hours the animals were sacrificed and their gastro-intestinal tracts were removed and dried. The dry tissue was then ground up and allowed to stand in

distilled water for 60 hours. After this period it was assumed that any inulin present would have been extracted. Quantitative samples of the extract were then counted by liquid scintillation.

The question of the role of the bladder in urine formation in these animals is critical. To test the possibility of prolonged bladder retention of urine, animals were injected with ^{22}Na and allowed to equilibrate for 24 hours. The animals were then placed in a small volume of water which was in turn pumped through a coil mounted in a well detector. This allowed continuous monitering of radioactivity on a rate meter equipped with a recorder. The degree of bladder retention of urine could then be inferred from the pattern of increase of radioactivity in the bath. That is, a linear increase would indicate little bladder retention while a step-wise increase would indicate intermittent urination with the urine being held in the bladder between urinations.

Another experiment was performed to determine the degree of reabsorption of water from urine forced to remain in the bladder. A group of animals previously injected with $^{14}COOH$-inulin was anesthetized and the cloaca of each was blocked. They were then placed in tap water for 12 hours. At the end of this period several animals were killed, an incision was made in the lower ventral abdominal region to expose the distended bladder and a urine sample was taken directly from the bladder. Inulin U/P values were then

determined for these animals. The remaining animals were left in their containers and the concentration of the bath was increased to 200 mOsm with sucrose. For the next 12 hours animals were killed and bladder urine:plasma inulin values were measured every four hours. An increase in inulin U/P is interpreted as an increase in water reabsorption.

Estimation of Mean Plasma Inulin Concentration from a Single Terminal Plasma Sample

During relatively long clearance periods the plasma concentration of inulin may decrease significantly as inulin is removed by the kidneys. A knowledge of the inulin space can be employed to estimate the mean plasma concentration when the final plasma concentration and the amount of inulin excreted are known. The relationship is as follows:

$$P_o = \frac{(P_f)\ (S_f) + E}{S_o} \qquad (4)$$

Where: P_o = Plasma inulin concentration at the beginning of the clearance period (DPM/ml)

P_f = Terminal plasma inulin concentration (DPM/ml)

S_o = Inulin space at the beginning of the clearance period (ml)

S_f = Inulin space at the end of the clearance period (ml)

E = Amount of inulin excreted during the clearance period (DPM)

Thus the mean plasma inulin concentration ($\overline{P}$) during the clearance period can be calculated as follows:

$$\overline{P} = \frac{P_o + P_f}{2} \qquad (5)$$

There is, however, an error associated with this method. It has been shown by Schachter, Freinkel and Schwartz (1950) that following a single intravenous inulin injection the concentration in the plasma and the interstitial fluids are equal at only one instant. That is, after the injection into the blood the inulin enters the interstitial space at the same time that it is being removed by the kidneys. Immediately after the concentrations in the plasma and the interstitial fluids equalize, the plasma concentration begins to decrease below that of the interstitial fluid because of the renal excretion of inulin. The use of a single subcutaneous injection of inulin, as was done here, presumably leads to a situation where the interstitial fluids are slightly more concentrated than the plasma. Thus the estimate of

P_o and, consequently $\bar{P}$, is slightly high. Since the time elapsed between the injection of inulin and the clearance period is relatively long, the gradient between the interstitial fluid and the plasma is fairly small. Additionally, the calculated mean plasma concentrations were only 10 - 15 percent above the terminal inulin concentrations. This represents the upper limit of possible error. In any event I feel that this error is more acceptable than the alternative which is to take more than one blood sample. These animals are sensitive to handling and even more critically, the taking of sequential blood samples would probably affect cardiovascular dynamics which have a pronounced effect on the kidney.

Radioinulin and Glofil I - 125 as GFR Markers

The clearance of ^{14}COOH-inulin and glofil I-125 were each determined simultaneously with the clearance of unlabeled inulin (Sigma Chemical Company). The "free-flow" method (equation 3) was used.

Ten mg of unlabeled inulin along with 1 μCi of either ^{14}COOH-inulin or glofil-^{125}I were injected subcutaneously in 0.1 ml amphibian Ringer's solution into anesthetized A. gracile larvae. The animals were allowed to equilibrate 18 hours (inulin) or 24 hours (glofil) before the GFR determinations were made.

Inulin clearances were measured on larvae immersed in tap

water and in an isosmotic solution of potassium sulfate (200 mOsm).

GFR in Free-Swimming Animals

Glomerular filtration rate in unanesthetized, unrestrained animals was measured by the "free-flow" method described above (equation 3). Animals were injected subcutaneously with ^{14}COOH-inulin (1 μCi) and, after the appropriate equilibration period, they were placed in individual containers holding 100 ml of tap water, 100 mOsm, 200 mOsm or 300 mOsm sucrose were used. In most experiments the animals were first equilibrated for four hours in the bath in which GFR was to be measured. In a few experiments, however, the animals were all started in tap water and the concentration of the medium was later increased by suddenly introducing sufficient 1 molal sucrose to bring the concentration to the desired level. Bath samples were taken at specified intervals over a period of several hours. At the end of the experiment a terminal plasma sample was taken and the GFR was determined (equations 3, 4 and 5).

Renal Tubular Function

A second means of assessing renal function involving direct urine collection was employed. Salamanders previously injected with ^{14}COOH-inulin were anesthetized and their bladders emptied by suprapubic compression. A small rubber bag with the bottom

cut out was then drawn over the head and forelimbs of each animal and tied immediately anterior to the hind limbs. Another hole was cut so that the tail could pass out of the bag. The bag was then sealed around the tail with a second ligature. At this point the open end of the bag was ventral to the cloaca. The inside of the bag was then thoroughly rinsed with distilled water and dried with chemically clean wipes. At this point the open end was closed with a third ligature and the animal was placed in 100 ml of either tap water or 200 mOsm sucrose. Animals placed in 200 mOsm sucrose were acclimated to this concentration for four hours prior to beginning the experiment. After six - twelve hours the urine was removed from the bags to a tared vial and weighed. A blood sample was also taken with a heparinized (NH_4^+ - heparin) blood collecting tube. The plasma was separated by centrifugation. To check the leakage of urine from the bags during the course of the experiment the bath was monitored for radioactivity.

To determine if a significant amount of water was reabsorbed from the urine across the skin, several animals were prepared with bags which were tied to their midsections in such a way that their cloacae did not open into the bags. A solution approximately equal in total solute concentration to urine (20 mOsm sucrose) and which contained a known concentration of inulin was then placed in the bag. After 12 hours the inulin concentration in the bags was

measured. An increase in concentration would indicate that water was being absorbed across the skin.

The "clearance" of any solute which appears in the urine can be determined from:

$$C_s = \frac{U\dot{V}}{P} \qquad (6)$$

This clearance can then be compared with the clearance of inulin which is equivalent to the GFR. If the clearance of the substance exceeds the GFR the substance must be secreted, whereas if C_s is less than the GFR the solute must be reabsorbed.

A more direct evaluation of the extent of net reabsorption or secretion of a given solute is provided by the relation:

$$X = (P_s)\ (GFR) - (U_s)\ (\dot{V}) \qquad (7)$$

Where X = net quantity of substance (S) secreted or reabsorbed

P_s = plasma concentration of S

U_s = urine concentration of S

$\dot{V}$ = rate of urine production

The urine collection bag method permits a direct estimate of $\dot{V}$ and provides urine for chemical analysis. From this data, clearance of water and various solutes can be assessed.

Osmolar clearance is the volume of isosmotic (to plasma) fluid cleared by the animal and is calculated as follows:

$$C_{osm} = \frac{U_{osm}}{P_{osm}} \dot{V} \qquad (8)$$

Where: C_{osm} = Osmolar clearance (ml/hr)

U_{osm} = Total solute concentration of urine (mOsm)

P_{osm} = Total solute concentration of plasma (mOsm)

$\dot{V}$ = Urine production rate

Free water clearance is the rate of excretion of osmotically free water and is the difference between $\dot{V}$ and C_{osm}.

Glomerular Activity

Glomerular activity is defined as the fraction of gomeruli actively filtering plasma at any one time. Decreases in glomerular activity accompanying decreases in GFR have been reported for the bullfrog (Forster, 1942), the rat (Van Liew, Deetjen and Boylan, 1967) and the dog (Keys and Swanson, 1971) among other animals. The technique employed in these studies has been to elevate the plasma glucose concentration to a level at which the glucose reabsorption process in the nephric tubules is saturated and glucose is being

reabsorbed at a maximal rate. This maximal rate is defined as the tubular maximum for glucose (Tm_G), and is given by:

$$Tm_G = L_G - E_G \qquad (9)$$

Where: L_G = GFR x plasma glucose concentration (mg/hr.) and is the filtered load

E_G = $\dot{V}$ x urine glucose concentration (mg/hr.) and is the amount excreted

It has been argued by Forster (1942) and others that if the plasma concentration for glucose (P_G) is above the level at which Tm_G is reached a decrease in Tm_G with decreasing GFR is an indication of decreasing glomerular activity. This is because the only way to decrease Tm_G when P_G is above threshold would be to decrease the number of reabsorptive sites for glucose (i.e. - decrease the number of functional nephric tubules).

An experiment using the urine collection bag technique was designed to determine if glomerular intermittency occurs in urodeles. Because of the necessity to ensure that plasma glucose concentration was relatively constant during the collection period large D. ensatus neotenes were used. This enabled the collection of blood samples both at the beginning and end of the experiment.

Animals previously injected with 2 μCi of ^{14}COOH-inulin

(19 hour equilibration period) and 0.1 ml/10g of 30 percent glucose (3 hour equilibration period) were anesthetized in Finquel. A blood sample was taken by syringe from a branchial vessel and the urine collection bag was tied in place. The animals were then placed in 150 ml of tap water, 50 mOsm mannitol or 100 mOsm mannitol. Mannitol was used because it would not interfere with the glucose assay if any leaked into the collection bags.

After approximately four hours the animals were sacrificed, the urine collected and weighed and a terminal blood sample taken. Urine inulin and glucose concentrations were determined as above. Plasma inulin and glucose concentrations were calculated as the mean of the initial and terminal concentrations of these substances.

Sodium Efflux

A set of experiments was performed to partition efflux into total and extra renal components. The renal component could then be determined by subtraction.

A series of animals was injected with $^{22}NaCl$ (2 μC in 0.1 ml amphibian Ringer's) and allowed to equilibrate 24 hours. The animals were then placed in individual containers in either tap water or 200 mOsm sucrose. The animals in the 200 mOsm had previously been allowed to equilibrate for four hours at that concentration. The bath (volume = 100 ml) was then sampled over the following twelve

hours and the efflux of ^{22}Na was determined. After this initial measurement was made the animals were anesthetized and separated into two groups. The cloacae of one half of the animals was blocked. The other half received sham ligatures. Next the animals were returned to the appropriate bath concentration and a second measurement of ^{22}Na efflux was made. The animals were then sacrificed and the specific activity of ^{22}Na in the plasma determined. Sodium efflux was calculated under each condition using the following relationship:

$$M_{out} = \frac{ds/dt}{SA} \qquad (10)$$

Where: M_{out} = Efflux (μeq Na/hr.)

ds/dt = rate of loss of ^{22}Na (CPM/hr.)

SA = specific activity of plasma (CPM/μeq Na)

Long Term Effects of Storage in 200 mOsm Sucrose

Two groups of animals were kept in individual containers for 13 days. One group was bathed in tap water while the other was in 200 mOsm sucrose. The groups were handled identically and had their water changed every second day. They were weighed daily between 3 and 5 P. M.

At the end of this period the animals were sacrificed and their

kidneys were removed and assayed for Na^+, K^+-Adenosine triphosphatase. The isolation and assay of this enzyme were done by the procedure of Boonkoom and Alvarado (1971). A brief description of this procedure follows.

The kidneys were chilled on ice in a small volume of the homogenizing medium (0.1% deoxycholate in 0.25 m sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA)). The tissues were then homogenized with a chilled Potter-Elvehjem glass homogenizer. The homogenate was centrifuged for 10 minutes at 10,000 x g at 2° C. The final pellet was then resuspended in 1 mM EDTA and was ready for assay.

The assay of enzyme activity was accomplished by measuring total ATPase activity (Mg^{++}-ATPase + Na^+, K^+-ATPase) in one set of test tubes and Mg^{++}-ATPase alone in another set of test tubes containing ouabain (10^{-4} m). Ouabain is known to inhibit Na^+, K^+-ATPase completely. The activity of the Na^+, K^+-ATPase is then obtained by subtraction. The assay medium contained 1 mM EDTA, 5 mM $MgCl_2$, 3 mM ATP, 100 mM NaCl, 20 mM KCl, 50 mM tris-HCl (pH 7.5) and 0.2 ml of the enzyme-containing solution. The assay was carried out for 20 minutes at 25° C. The reaction was then stopped by the addition of 1 ml of 20 percent trichloroacetic acid. The protein precipitated by this procedure was then removed by centrifugation. Finally an aliquot of the supernatent was

analysed for phosphate by the method of Stanton (1968). Protein in the enzyme-containing solution was determined by the method of Lowry et al. (1951).

RESULTS

Simultaneous Clearance of Radioactive Compounds And Unlabeled Inulin

Experiments were conducted to assess the reliability of two radioactive tracers as GFR markers. Table 1 gives the simultaneous values of GFR using the two markers (either ^{14}COOH-inulin vs. unlabeled inulin or ^{125}I-glofil vs. unlabeled inulin) for each animal examined. The ratios of the two GFR values (± 95% confidence intervals) are also given. In considering these ratios the 95 percent confidence intervals (C.I.) about the means include a ratio of one in both cases. These are 1.07 ± 0.28 for ^{14}COOH-inulin vs. unlabeled inulin and 1.36 ± 0.48 for ^{125}I-glofil vs. unlabeled inulin. This indicates that the clearance of the radioactive markers did not significantly differ from that of the unlabeled inulin. Furthermore, if the regression of Y on X is computed ($GFR_{\text{radioactive tracer}}$ vs. $GFR_{\text{unlabeled inulin}}$) the slope is 1.19 ± 0.76 (95% C.I.) in the case of ^{14}COOH-inulin. The comparable slope for ^{125}I-glofil vs. unlabeled inulin is 0.72 ± 0.38 (95% C.I.). In neither case is the slope significantly different than one, providing further evidence that these radioactive tracers did

Table 1. Simultaneous measurements of either ^{14}COOH-inulin or ^{125}I-glofil and unlabeled inulin. The weight of the animals was 10.1 ± 0.8 g (mean ± SE). All measurements were performed in the summer on A. gracile.

Radioactive compound being tested	GFR (ml/10g-hr.)		Ratio GFR_1/GFR_2
	Radioactive Tracer (1)	Unlabeled Inulin (2)	
^{14}COOH-inulin	0.136	0.090	1.51
	0.100	0.098	1.02
	0.108	0.103	1.05
	0.097	0.070	1.38
	0.039	0.051	0.76[a]
	0.033	0.066	0.50[a]
	0.015	0.014	1.07[a]
	0.047	0.037	1.27[a]
$\bar{X}$ ± 95% Confidence Interval			1.07 ± 0.28
^{125}I-glofil	0.018	0.028	0.64
	0.231	0.198	1.17
	0.193	0.108	1.79
	0.162	0.081	2.00
	0.239	0.198	1.21
	0.199	0.091	2.19
	0.099	0.099	1.00
	0.342	0.405	0.84
$\bar{X}$ ± 95% Confidence Interval			1.36 ± 0.48

[a] GFR measured in 200 mOsm potassium sulfate.

not give significantly different values for GFR than did unlabeled inulin.

The computation of a correlation coefficient for GFR as measured by ^{14}COOH-inulin versus GFR as measured by unlabeled inulin was also performed. It was found that the two clearances correlated quite well over a wide range of GFR values observed ($R = 0.85$).

The validity of the use of ^{14}COOH-inulin as a GFR marker has been previously established in the rat (Maude et al., 1965) and in Necturus (Tanner and Klose, 1966). It therefore seems that this compound (^{14}COOH-inulin) is a reliable index of GFR in these animals.

Although ^{125}I-glofil was not used in other experiments its clearance did not differ significantly from that of unlabeled inulin. The discrepancy between glofil and inulin was greater than that noted between the two inulins, however. It has been reported that ^{125}I-glofil is a reliable GFR marker in humans (Elwood et al., 1967) but it appears to underestimate GFR in dogs (Gagnon et al., 1971). In view of the results reported here it would seem that the clearance of ^{125}I-glofil in amphibians needs further investigation before it is put into use as a GFR marker in these animals.

Extra Renal Loss of ^{14}COOH-Inulin and ^{125}I-Glofil

Extra renal losses of inulin and glofil are very low. Three D. ensatus larvae whose cloacae had been blocked lost ^{14}COOH-inulin at a rate of 0.04 μg inulin/10g-hr. This is one - five percent of the rate of loss of inulin in animals whose cloacae are open. Similar results were obtained when A. gracile larvae were tested for extra renal loss of glofil. It was found that three animals lost an average of 14.7 CPM/10g-hr. This is less than two percent of the rate of renal loss of this compound. Since it is almost impossible to completely block the cloaca, the slight loss of each compound in ligatured animals probably represents leakage and/or bleeding associated with the stitching.

Drinking Rates

Dicamptodon ensatus larvae were placed in solutions containing approximately 10^6 CPM of ^{14}COOH-inulin. Table 2 shows that no significant drinking occurs. These animals occasionally gulp air and it is possible that a little water enters their gastro-intestinal tract at this time.

Table 2. Rate of drinking in D. ensatus larvae. Values are expressed as mean ± SE (N). The mean weight of the animals in the tap water group was 8.2 ± 0.9 g. The mean weight of those in the 200 mOsm group was 7.0 ± 0.5 g.

	In Tap Water	In 200 mOsm Sucrose
Drinking Rate (μl/10g-hr.)	1.12 ± 0.71 (5)	0.68 ± 0.29 (5)

Pattern of Urination

The efflux of ^{22}Na from A. gracile larvae was continuous indicating little retention of urine in the bladder (Figure 1a). This pattern was observed over several hours on each of four larvae. In contrast Figure 1b is a recording of the increase in radioactivity in bath containing an A. gracile adult previously injected with ^{22}Na. Adults presumably do store urine in their bladders and thus urinate intermittently. It is interesting to note that ^{22}Na efflux is very low except during urination. This would indicate that extra renal loss of this ion is very low in adults. A similar pattern has been reported in Rana pipiens (Greenwald, 1971).

Effect of Forced Storage of Urine in the Bladder

Several A. gracile with ligatured cloacae were kept in tap water for twelve hours. The urine produced during this time was then forced into the bladder and hind gut. Bladder urine was sampled

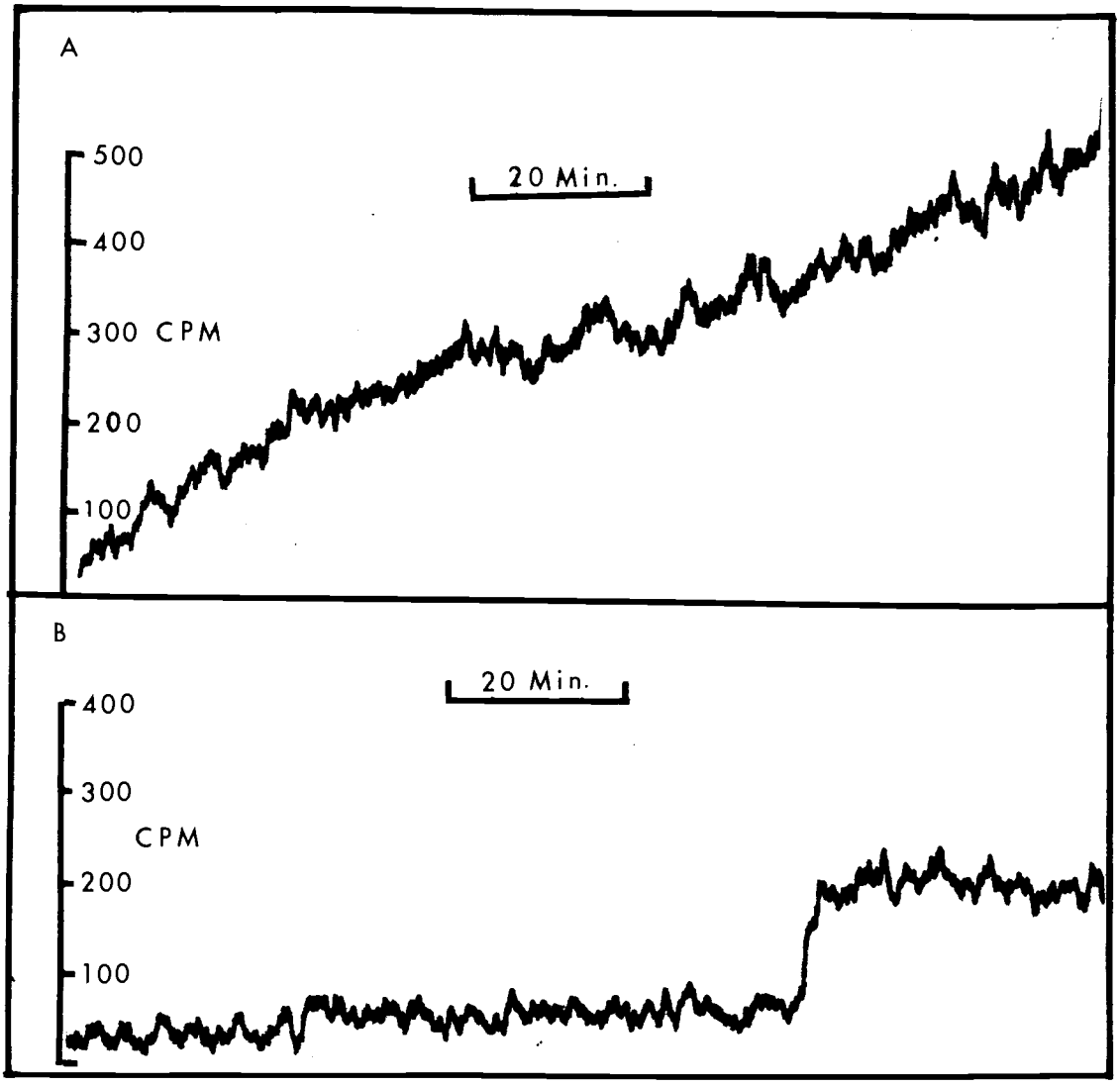

Figure 1. A. Typical recording of the increase in radioactivity of the bath due to the efflux of ^{22}Na by a larval Ambystoma gracile. The time constant on the rate meter was 50 seconds. The animal weighed approximately 10g. B. Recording of the increase in radioactivity of the bath due to the efflux of ^{22}Na by an adult Ambystoma gracile. The time constant on the rate meter was 50 seconds. The animal weighed approximately 10g.

at the end of this period and the mean U/P value of (1.3 ± 0.2 (3)) was not significantly different from the mean ratios obtained from animals which were allowed to void their urine directly into urine collection bags, thus presumably bypassing the bladder (U/P = 1.4 ± 0.1 (19)). Apparently very little, if any, reabsorption of water takes place in the bladder of larvae in tap water. A second group of animals were treated exactly the same way except that instead of sampling their bladder urine at the end of the 12 hours in tap water the concentration of the bath was increased to 200 mOsm with sucrose. At four hour intervals one animal was killed and bladder urine and plasma samples were obtained. It can be seen that over this period the U/P inulin values were quite variable but did not increase enough to indicate significant reabsorption of water by the bladder (Figure 2). In fact two out of the three animals gave U/P values less than one.

Measurement of Inulin Space

Figure 3 depicts plots of the time courses of the volumes of distribution for inulin in A. gracile and D. ensatus. In both cases the inulin space initially decreases to a minimum. This is followed by a rise in the inulin space of D. ensatus over the next several hours at a rate of 0.26 ml/10g-hr. (F-test for linearity). The inulin space in A. gracile remained essentially constant over the period it was

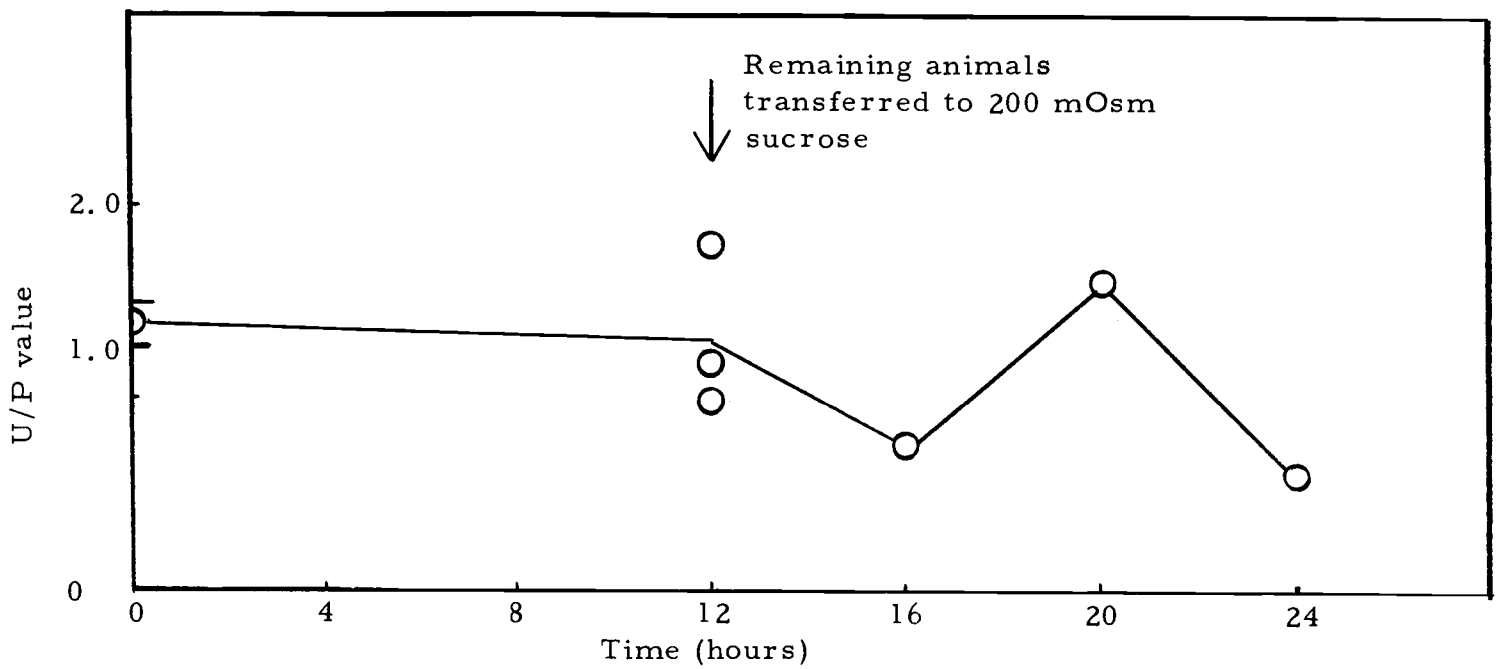

Figure 2. Effect of forced bladder storage on urine concentration. The point at time zero is the mean (± SE) of inulin U/P values obtained from A. gracile urine collected in bags and represents the U/P values of animals permitted to void their urine spontaneously. The three points at 12 hours are U/P values from animals forced to store urine in their bladders for up to 12 hours. From 12 hours on each point represents the U/P value of an animal which produced and stored urine in its bladder for 12 hours prior to being transferred to 200 mOsm sucrose.

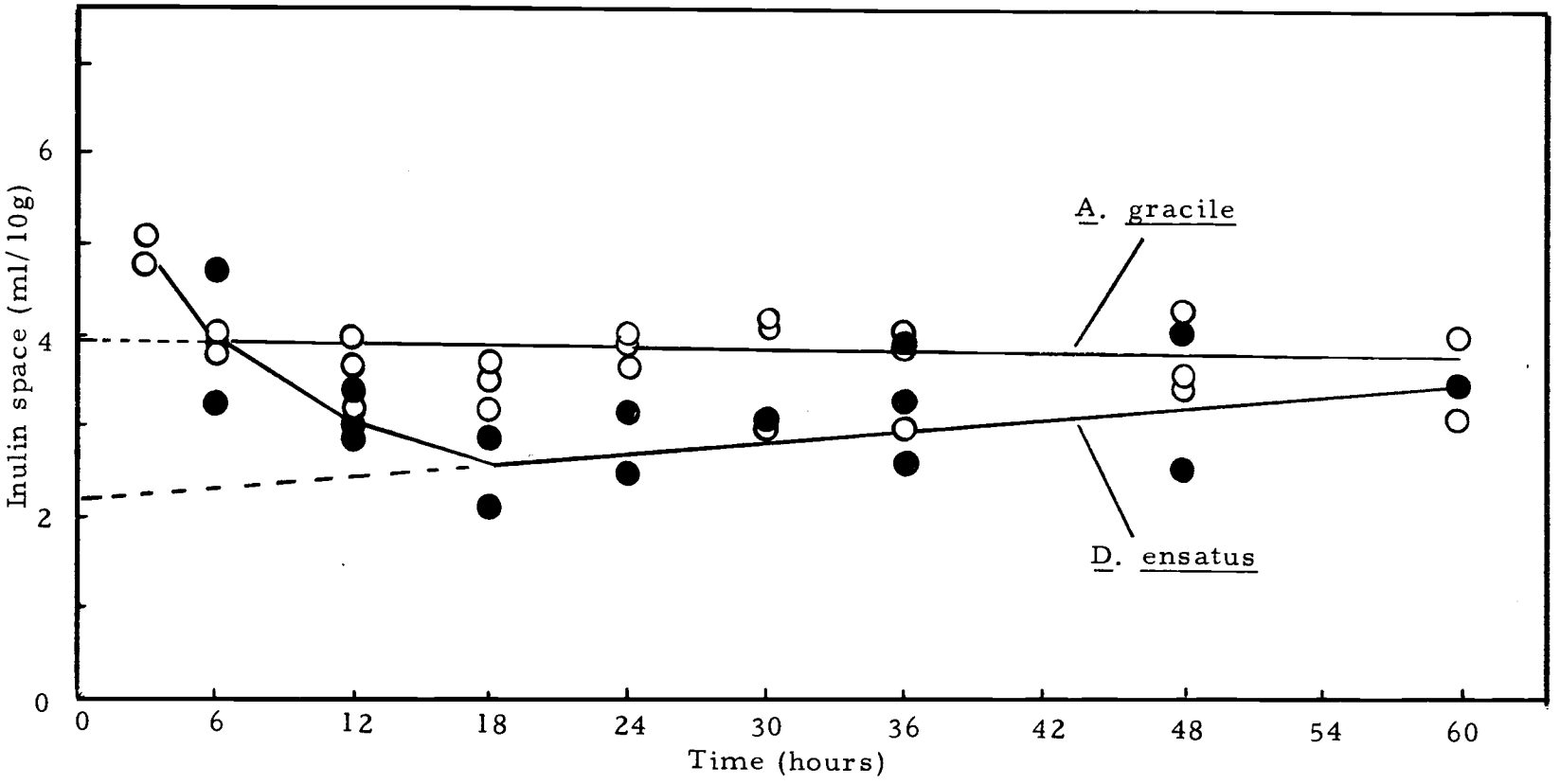

Figure 3. Inulin space as a function of time. Each point represents the inulin space of an individual animal at the indicated time after the subcutaneous injection of inulin. The solid line in the linear range was obtained by regression analysis. The y-intercept, obtained by extrapolation (dotted line) is a measure of the extracellular space. Open circles are A. gracile. Closed circles are D. ensatus.

monitored after reaching the minimum volume. The space obtained by extrapolating the linear portion of the curve back to zero time is an estimate of the extracellular space (Thorson, 1964). The spaces for D. ensatus and A. gracile were 2.27 ml/10g and 3.86 ml/10g respectively. Published values for extra-cellular fluid volume in urodeles using the sucrose space are as follows: Necturus maculosus, 2.41 ml/10g; Cryptobranchus alleganiensis, 2.20 ml/10g; and Amphiuma means tridactylum, 2.18 ml/10g (Thorson, 1964). The space reported here for A. gracile is thus significantly larger than that reported for other urodeles. This, however, is not too surprising as it was repeatedly observed in the laboratory that larger blood samples were obtainable from A. gracile larvae than from D. ensatus larvae of comparable size.

The time required for the space to reach a minimum value can be taken as the equilibration period for the inulin. It can be seen that this period is much shorter in A. gracile (12 hours) than in D. ensatus (18 hours).

Measurement of GFR by the "Free-Flow" Method

The results of GFR measurements under "free-flow" conditions are given in Table 3. In general winter animals tended to have lower values for GFR than did spring and summer animals. The difference between winter and spring A. gracile larvae was

significant ($P < 0.05$). The difference between winter and summer animals, although appearing large, is only marginally significant ($P < 0.10$). This is the result of very great variability in the summer-fall group. A similar trend toward lower GFR in winter animals has been reported for Bufo arenarum (Uranga, 1958).

Table 3. GFR (mean ± SE (N)) in larval salamanders immersed in tap water. Values are listed according to the season during which they were obtained. The A. gracile weighed 11.7 ± 0.5 g. The D. ensatus weighed 12.3 ± 0.9 g.

	GFR (ml/10g-hr.)		
	Winter	Spring	Summer-fall
A. gracile	0.056 ± 0.016 (5)	0.124 ± 0.012 (5)	0.107 ± 0.017 (10)
D. ensatus	----	----	0.166 ± 0.019 (9)

The rate of glomerular filtration as measured here for spring and summer-fall A. gracile and summer-fall D. ensatus lies within the range of previously reported values within this group. Alvarado and Johnson (1965) report a GFR of 0.25 ml/10g-hr. in Ambystoma tigrinum. Parsons and Alvarado (1968) have measured GFR in A. gracile and found this to be 0.33 ml/10g-hr. Kirschner et al. (1970) report a GFR of 0.083 ml/10g-hr. in A. tigrinum. As season was not considered in each of these studies it is difficult to compare their data with that obtained here. It is interesting to note, however, that when all of the GFR data obtained here for tap water A. gracile

are pooled without regard to season the value is 0.098 ml/10g-hr. This is not significantly different from the GFR reported by Kirschner et al. (1970) of 0.083 ml/10g-hr. On the other hand, the values for GFR in spring and summer-fall animals of both species examined here are not much smaller than the value of 0.25 ml/10g-hr. reported by Alvarado and Johnson (1965). In fact, the GFR of D. ensatus is not significantly different than 0.25 ml/10g-hr. This serves to point out the need for the consideration of seasonal effects in measurements of this type on these animals.

The GFR values reported here and by Kirschner et al. (1971) tend to be lower than those of Alvarado and Johnson (1965) and Parsons and Alvarado (1968). This might be related to the method of administration of inulin in the various investigations. In the two earlier studies the inulin was injected intraperitoneally while in the study of Kirschner et al. (1971) inulin was injected subcutaneously as was the case here. It has been reported that eleven percent of the "filtrate" is nephrostomal in origin in Necturus (White, 1929). If this is the case in Ambystoma intraperitoneal injection might lead to slightly high estimates of GFR.

Effect of Decreasing the Osmotic Load on GFR

Figure 4 depicts the effect of increased external osmotic concentration on GFR in the two species. It can be seen that A. gracile

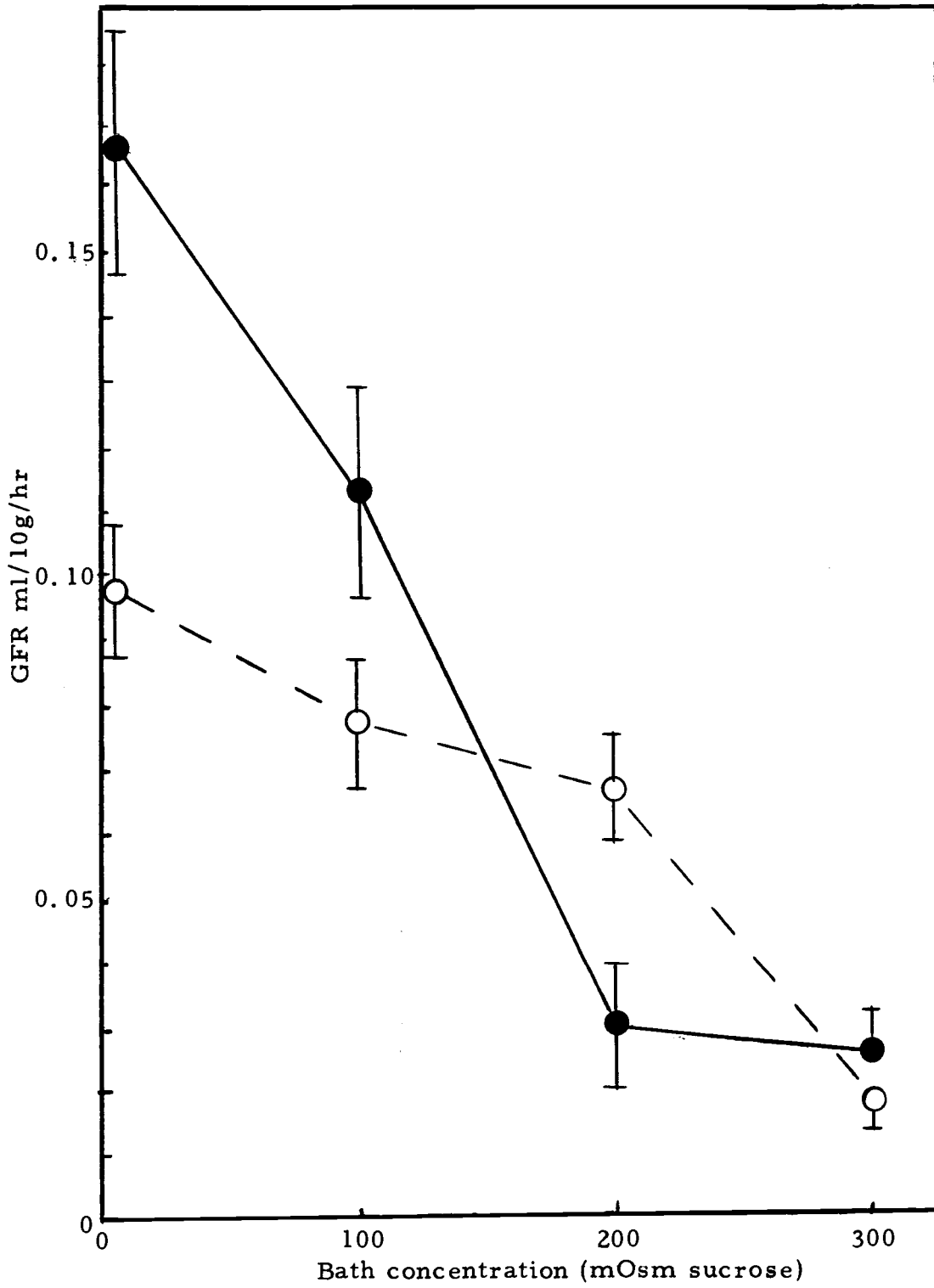

Figure 4. Glomerular filtration rate as a function of external osmotic concentration. Each point is the mean ± the standard error. Open circles are A. gracile. Closed circles are D. ensatus.

larvae decrease their GFR more of less linearly between tap water and 300 mOsm sucrose. Dicamptodon ensatus larvae also show a linear decrease in GFR. However, this decrease is more precipitous up to 200 mOsm and levels off between 200 and 300 mOsm.

Figure 5 illustrates the results of a typical experiment in which four groups of D. ensatus were placed in tap water and inulin excretion was monitored. After 12 hours had elapsed one molal sucrose was added in sufficient quantity to bring the external sucrose concentration in groups 2, 3 and 4 to 100, 200 and 300 mOsm respectively. The rate of inulin excretion was reduced as a function of the final bath osmolality. It was further observed that the mean plasma concentrations of inulin at the end of the experiment were 9.7 μg/ml (tap water); 9.9 μg/ml (100 mOsm); 17.8 μg/ml (200 mOsm) and 22.6 μg/ml (300 mOsm). Since the inulin dose was uniform (approximately 0.1 μCi/g animal weight) and since the initial rates of inulin loss in tap water were quite uniform, these differences are significant. Obviously the animals in the higher sucrose concentrations were filtering at a much lower rate.

Figure 5 shows that the decrease in GFR occurs within a few hours of the increase in external osmolality. To better resolve the length of this latency a group of four D. ensatus larvae were sampled hourly after bringing the osmolality of the bath to 200 mOsm with sucrose. Figure 6 shows that a period of about two to three

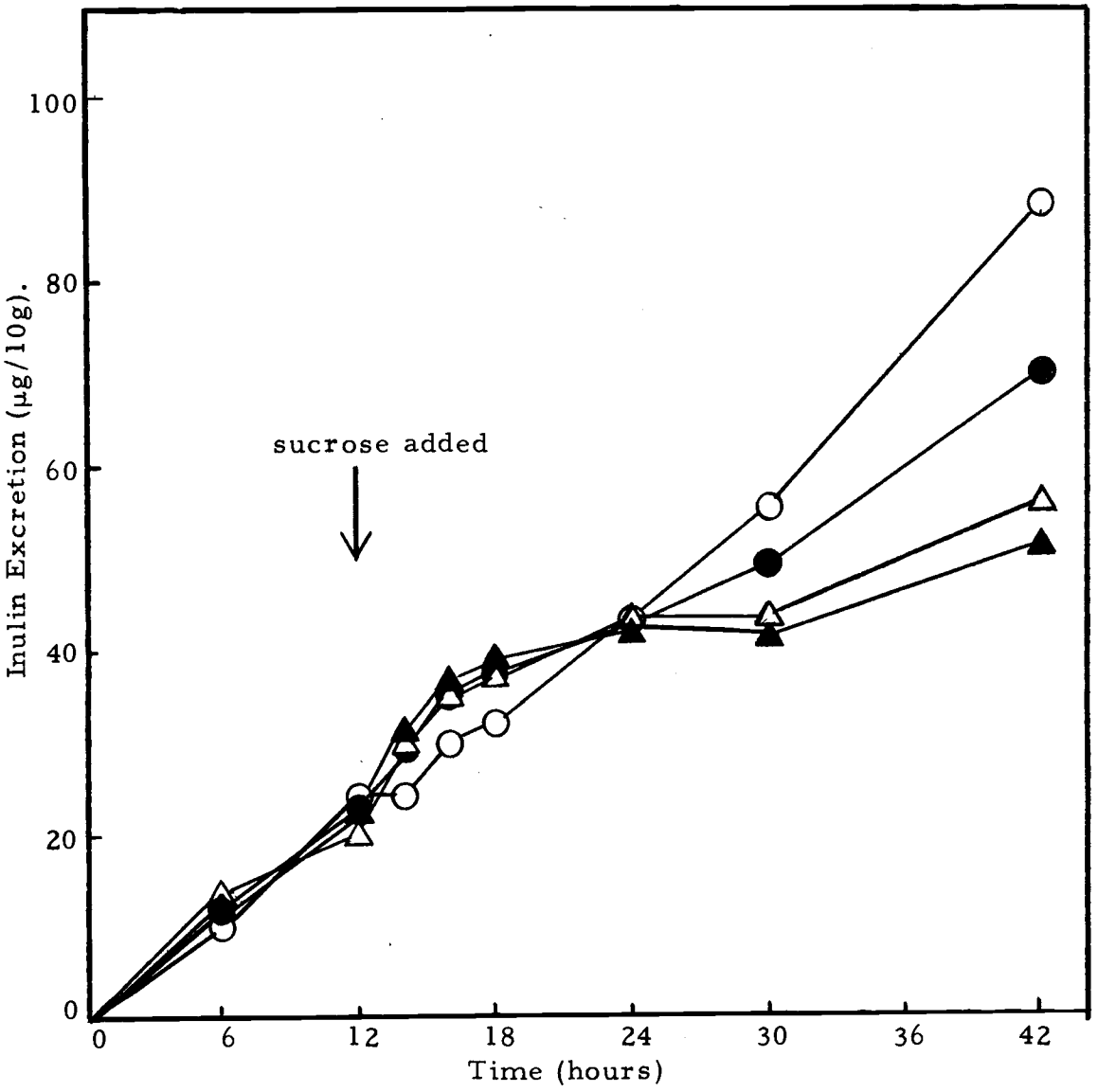

Figure 5. Inulin excretion as a function of time in D. ensatus. Each point represents the mean of 5 animals (except in 200 mOsm group, N = 4). The animals began the clearance in tap water. At 12 hours sufficient 1 m sucrose was added to bring the bath th the desired concentration (arrow): final bath concentrations in the groups are indicated by; ○: Tap water; ●: 100 mOsm; △: 200 mOsm; ▲: 300 mOsm.

hours is required for the decrease in GFR to occur.

Another experiment was devised to evaluate the time course of changes in GFR when animals are transferred from 200 mOsm sucrose to tap water. Seven D. ensatus larvae were equilibrated in 200 mOsm sucrose for four hours at which time sampling of the bath commenced. Six hours after the first sample was taken the 200 mOsm sucrose was removed by aspiration while tap water was simultaneously replacing it. In this way the animal was immersed continuously. The decrease in radioactivity in the bath to near background after this procedure insured that virtually all of the solute had been removed. It can be seen in Figure 7 that approximately five hours after the increase in osmotic load the mean inulin excretion rate for the group increased dramatically. The very large standard errors at the points following the increase serve as an indication of the variability seen both in GFR and in terms of the duration of the latency. Among the seven animals, three showed no increase until after the termination of hourly samples and, therefore, their latencies cannot be evaluated. Of the others, one responded after four hours, two after five hours and the final animal showed an increase at the sixth hour. I feel that five to six hours is a reasonable estimate of the recovery time. Regardless of the exact recovery time, it is clear that it is significantly longer than the time required for decreases of GFR in response to a decreased

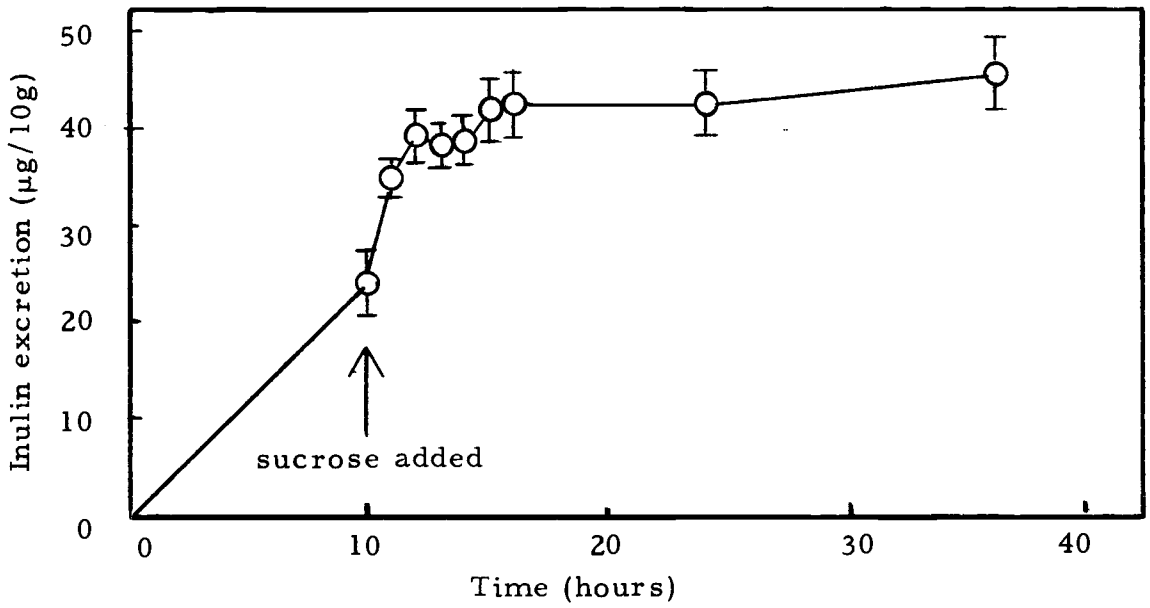

Figure 6. Inulin excretion as a function of time in D. ensatus. Each point is the mean ± SE of four animals. Sufficient 1 m sucrose added at 10 hours to bring bath concentration to 200 mOsm (arrow).

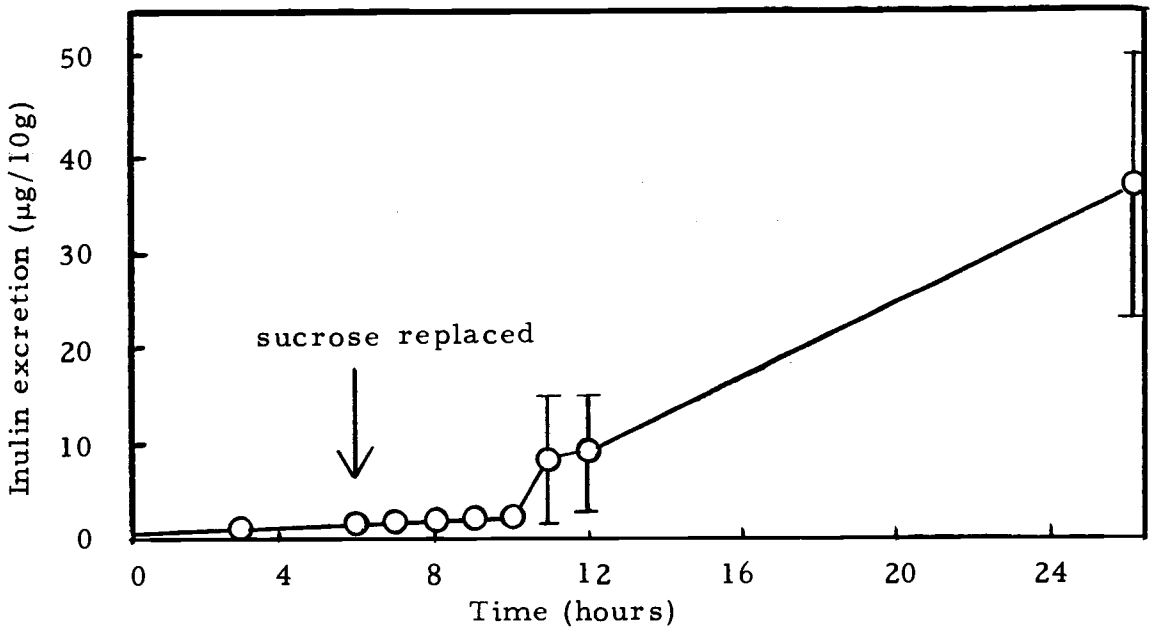

Figure 7. Inulin excretion as a function of time in D. ensatus. Each point is the mean ± SE of 7 animals. At 6 hours 200 mOsm sucrose was replaced with tap water (arrow).

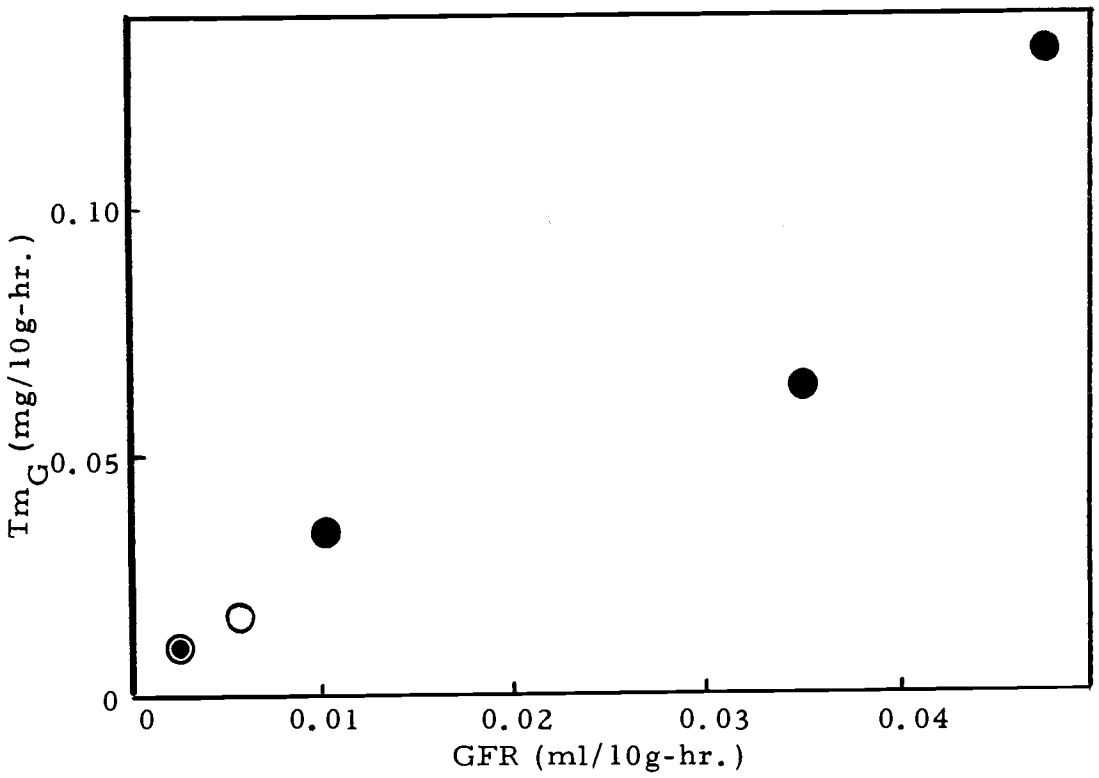

Figure 8. Maximal rate of glucose reabsorption (Tm_G) as a function of GFR. Each point represents one animal. ●: tap water animals; ◉: 50 mOsm mannitol animals; ○: 100 mOsm mannitol animals.

osmotic load. That the recovery was complete is evidenced by a GFR of 0.104 ± 0.012 ml/10g-hr. between six hours after elevation of the osmotic load and the end of the experiment fourteen hours later.

Tm_G Versus GFR as a Measure of Glomerular Activity

The relationship between maximal rate of tubular reabsorption of glucose (Tm_G) and GFR is a measure of glomerular activity (Forster, 1942). The determination of Tm_G for D. ensatus neotenes was carried out over a fairly wide range of glomerular filtration rates. Plasma glucose concentration was elevated in the animals to a point high enough to produce marked glycuresis. The mean plasma glucose concentration was 449 ± 45 mg/100 ml. The mean urine glucose concentration was 190 ± 36 mg/100 ml. Forster (1942) has determined that Tm_G is reached in bullfrogs when plasma glucose concentration exceeds 300 mg/100 ml. Assuming that D. ensatus neotenes are similar to bullfrogs in this regard the glucose reabsorptive process in these salamanders was operating at Tm. The very high urine glucose concentrations indicate that this was the case.

Figure 8 is a plot of Tm_G as a function of GFR in these animals. It would appear that decreases in GFR are brought about by decreases in glomerular activity in these animals.

Assessment of Renal Tubular Function

The results obtained with urine collection bags are subject to slight error due to leakage. The apparent outwardly directed component of this leakage was estimated by monitering the escape of inulin into the bath. When excessive escape of inulin into the bath occurred the animal in question was eliminated from further consideration. Generally this leakage was below 15 percent of the urine volume.

The appearance of inulin in the bath revealed little about inward leakage. I feel that this was negligible, however, as the value of $\dot{V}$ obtained by this method for A. gracile (0.077 ml/10g-hr.) agrees with the osmotic uptake of 0.086 ml/10g-hr. measured for this species at the same temperature by Alvarado and Dietz (1970a). Furthermore, the concentrations of various urine constituents measured by this method are slightly higher than previously reported urine concentrations determined on urine collected directly from animals of the same species (Parsons and Alvarado, 1968; Alvarado and Dietz, 1970). If significant leakage had occurred one would expect low values for urine concentrations.

In order to determine if these animals produced urine at a steady rate while the collection bags were attached, experiments of three different lengths were performed. The collection periods

lasted 6, 12 and 18 hours respectively. It can be seen in Figure 9 that urine production is relatively constant over the initial 12 hours and then decreases somewhat. It is difficult to determine whether this represents an initial diuresis or a later decrease in urine production, although the former is more likely in view of the stress involved with the use of the collection bags. To assure a uniform rate of urine production within each group, all experiments of this nature were limited to a 12 hour maximum duration.

The possibility of reabsorption of water across the skin from the urine in the bags was tested by tying on bags in such a way that the cloaca did not empty into them. A solution of 20 mOsm sucrose (which approximates urine total solute) containing a known amount of ^{14}COOH-inulin was then placed in the bag. The ratio of final inulin concentration to initial inulin concentration after 12 hours in seven A. gracile prepared in this manner was 0.98 ± 0.03, indicating no reabsorption of water from the bags. Bentley and Heller (1964) have used this method of urine collection on urodeles and also found no significant reabsorption of water across the skin.

The collection of urine permits the evaluation of several parameters including both tubular and glomerular phenomena. Tables 4 and 5 show the values in A. gracile and D. ensatus in tap water and 200 mOsm sucrose.

The values obtained by this method for GFR in A. gracile in

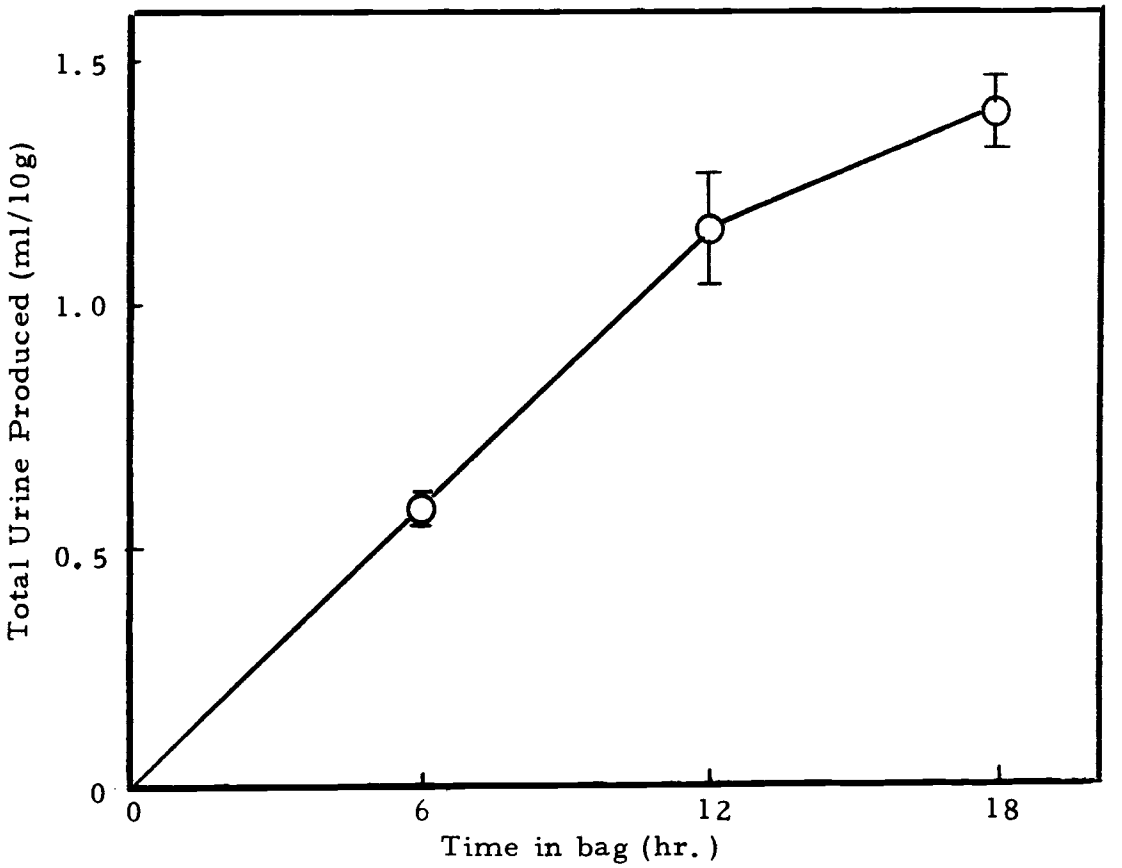

Figure 9. Volume of urine voided into urine collection bags as a function of time. Each point is mean ± SE. Species was A. gracile. N = 12 (6 hrs.); 8 (12 hrs.); 9 (18 hrs.).

Table 4. Renal function of A. gracile under normal and reduced osmotic load.

Parameter	Tap water	200 mOsm	Significance
$\dot{V}$ (ml/10g-hour)	0.077 ± 0.007 (19)	0.019 ± 0.002 (16)	**
U/P (inulin)	1.4 ± 0.1 (19)	1.7 ± 0.1 (16)	*
GFR (ml/10g-hour)	0.102 ± 0.007 (19)	0.031 ± 0.004 (16)	**
Osmolar clearance (ml/10g-hour)	0.014 ± 0.001 (19)	0.009 ± 0.000 (13)	**
Free water clearance (ml/10g-hour)	0.063 ± 0.006 (19)	0.011 ± 0.000 (13)	**

* Significantly different ($P < 0.05$).

** Significantly different ($P < 0.01$).

Table 5. Renal function of Dicamptodon ensatus under conditions of normal and reduced osmotic load.

Parameter	Tap water	200 mOsm	Significance
V (ml/10g-hour)	0.040 ± 0.003 (4)	0.003 ± 0.0008 (4)	**
U/P (Inulin)	1.4 ± 0.0 (4)	2.0 ± 0.1 (4)	**
GFR (ml/10g-hour)	0.056 ± 0.004 (4)	0.006 ± 0.001 (4)	*
Osmolar clearance (ml/10g-hour)	0.004 ± 0.000 (4)	0.001 ± 0.000 (3)	**
Free water clearance (ml/10g-hour)	0.035 ± 0.003 (4)	0.003 ± 0.000 (3)	**

* Significantly different ($P < 0.05$)

** Significantly different ($P < 0.01$)

tap water are in close agreement with those obtained by the "free-flow" method using spring animals of the same species (Table 3). However, in D. ensatus the filtration rate as determined by the use of urine collection bags (Table 5) is significantly lower ($P < 0.01$) than that determined with the "free-flow" method (Table 3). A possible explanation for this lies in the anatomical arrangement of the kidneys. In D. ensatus the kidneys are located at a point approximately dorsal to the cloaca. It is thus possible that the ligatures used to hold the collection bags in place acted to partially cut off circulation to the kidneys. The kidneys of A. gracile are positioned more anteriorly and thus would not be susceptible to this problem.

In both species the result of the decreased osmotic uptake of water is a reduction of GFR and a slight increase in tubular reabsorption of water as indicated by an increase in inulin U/P values. The net effects of these changes are decreases in urine production rate and free water clearances. The results of analyses of urine and plasma samples from both species are given in Tables 6 and 7. Urine concentrations were much lower for D. ensatus larvae than for A. gracile under similar conditions. These differences correlate with the environmental solute concentrations to which the two species are exposed in nature (Table 8).

Table 6. Composition of urine and plasma of A. gracile larvae. Values are expressed as mean ± SE (N). Significance determined with t-test.

Substance	Tap water animals		200 mOsm animals		$\frac{\bar{U}_2}{\bar{U}_1}$	Significance of difference (U_1 and U_2)
	Urine ($\bar{U}_1$)	Plasma	Urine ($\bar{U}_2$)	Plasma		
Total solute (mOsm/L)	36 ± 2 (19)	192 ± 2 (19)	86 ± 8 (14)	192 ± 2 (13)	2.4	**
Na^+ (μeq/ml)	10.4 ± 0.7 (19)	98 ± 1.5 (19)	26.9 ± 3.7 (15)	92.4 ± 1.1 (16)	2.6	**
K^+ (μeq/ml)	3.7 ± 0.4 (19)	9.9 ± 0.7 (19)	6.2 ± 0.9 (15)	7.4 ± 0.3 (17)	1.7	*
Cl^- (μeq/ml)	6.6 ± 0.4 (19)	76.7 ± 1.9 (15)	17.3 ± 2.2 (15)	72.5 ± 1.2 (17)	2.6	**
$PO_4^=$ (μAtm P/ml)	2.69 ± 0.22 (14)	3.31 ± 0.21 (14)	----	----	---	
Urea (μAtm N/ml)	1.68 ± 0.29 (13)	1.66 ± 0.37 (14)	2.07 ± 0.66 (5)	2.05 ± 0.62 (5)	1.23	
NH_3 (μAtm N/ml)	2.21 ± 0.33 (10)	----	3.26 ± 0.22 (3)	----	1.48	

* $P < 0.05$

** $P < 0.01$

Table 7. Composition of urine and plasma of D. ensatus larvae. Values are expressed as mean ± SE (N). Significance determined with t-test.

Substance	Tap water animals		200 mOsm animals		$\frac{\bar{U}_2}{\bar{U}_1}$	Significance of difference (U_1 and U_2)
	Urine ($\bar{U}_1$)	Plasma	Urine ($\bar{U}_2$)	Plasma		
Total solute (mOsm/l)	20 ± 2 (4)	192 ± 3 (4)	53 ± 3 (4)	207 ± 5 (3)	2.6	**
Na^+ (μeq/ml)	5.2 ± 0.4 (4)	89.5 ± 2.6 (4)	15.2 ± 2.7 (3)	92.1 ± 6.7 (3)	2.9	**
K^+ (μeq/ml)	2.8 ± 0.4 (4)	15.8 ± 1.6 (4)	5.2 ± 0.3 (2)	15.8 ± 0.8 (3)	1.8	**
Cl^- (μeq/ml)	1.8 ± 0.1 (4)	72.1 ± 0.8 (4)	10.52 ± 2.1 (3)	70.4 ± 6.4 (2)	5.8	**

** $P < 0.01$

Table 8. Concentration of dissolved solutes in habitats typical of A. gracile and D. ensatus.

Substance	Owl Creek (A. gracile)	Parker Creek (D. ensatus)
Total solute (mOsm/l)	5	1
Sodium (mM/l)	0.4	0.1
Chloride (mM/l)	0.1	<0.1

Total solute in the urine increased markedly in both species when animals were placed in 200 mOsm sucrose. Published examples of urine total solute in other amphibians adapted to tap or pond water range from 25.9 mOsm in R. esculenta (Mayer, 1969) to about 75 mOsm in A. tigrinum larvae (Alvarado and Kirschner, 1963). Increases in urine total solute in animals subjected to concentrated media have been noted in several amphibian species including Xenopus laevis (McBean and Goldstein, 1970), Rana esculenta (Mayer, 1969), Rana cancrivora (Gordon et al., 1961) and Bufo viridis (Gordon, 1962).

Sodium, potassium and chloride concentrations measured in the urine of these two species were slightly higher ($P < 0.01$) than most previously published values in this family of urodeles (Alvarado and Kirschner, 1963; Parsons and Alvarado, 1968; Alvarado and Dietz, 1970a). As with total solute, the concentrations of each of the

ions approximately doubled in animals subjected to sudden increases in external osmotic concentration. Increases in urinary salt concentration have been previously noted in A. tigrinum subjected to elevated external salt concentration (Kirschner et al., 1971). To my knowledge the present finding is the first report of such an increase when osmotic concentration was increased with a non-electrolyte.

Phosphate concentrations were determined on urine from tap water A. gracile only (Table 6). The values are somewhat higher than have been previously reported. Walker and Hudson (1937b) report urinary phosphate concentrations of below 1 μAtm P/ml for Necturus maculosus. This is about one third of the value determined here.

The results of the analyses of urinary ammonia and urea concentrations in A. gracile urine were quite variable. Similar variability has been noted previously in amphibians (Balinsky and Baldwin, 1961; Cragg, Balinsky and Baldwin, 1961; Fanelli and Goldstein, 1964). Excretion rates for ammonia and urea in A.gracile were 0.17 and 0.13 μAtm N/10g-hr. respectively. Thus about 57 percent of the urinary nitrogen is in the form of ammonia. This is in very close agreement with the findings of Fanelli and Goldstein (1964) for N. maculosus which excretes urinary ammonia at a rate of 0.18 μAtm N/10g-hr. and urea at the rate of 0.12 μAtm N/10g-hr. A few urea and ammonia concentrations were determined for urine

from 200 mOsm animals. As with the other solutes measured there was an increase in concentration of these two products. The small number of determinations, however, prevents the assignment of much significance to this increase.

Plasma concentrations of the various substances assayed are given in Tables 6 and 7. The total solute, sodium and chloride concentrations did not vary much between the two species examined here and are in close agreement with previously reported concentrations of the substances in urodeles (Shipp et al., 1958); Alvarado and Kirschner, 1963; Alvarado and Dietz, 1970a). Plasma potassium concentrations as measured here were 9.9 μeq/ml for A. gracile and 15.8 μeq/ml for D. ensatus in tap water. The latter value is undoubtedly high probably because of hemolysis. The value for A. gracile agrees reasonably well with the value of 8 μeq/ml obtained previously for the same species (Alvarado and Dietz, 1970a). It is somewhat higher than values reported for A. tigrinum (5.4 μeq/ml; Alvarado and Kirschner, 1963) and for N. maculosus (3.2 μeq/ml; Shipp et al., 1958).

The results of the determination of plasma phosphate concentration, like those of urine phosphate, were somewhat higher than have been reported for Necturus maculosus (Walker and Hudson, 1937b; Shipp et al., 1958). Necturus is an obligate neotene while the larval A. gracile investigated here were probably in the early

stages of metamorphosis. During metamorphosis there is a great deal of osteogenesis occurring in urodeles. This requires large amounts of phosphate. It is not unreasonable to expect that plasma and urine phosphate levels would be relatively high under such conditions.

Plasma urea concentrations determined here for A. gracile larvae averaged 1.66 μAtm N/ml for animals in tap water. Walker and Hudson (1937a) report urea levels in N. maculosus plasma of about 1.54 μAtm N/ml. There was a slight increase in urea plasma concentration in the few 200 mOsm animals examined.

It is possible to estimate the volume of water reabsorbed by the kidney tubules as the difference between the volume of plasma filtered and the volume of urine produced. Although A. gracile subjected to 200 mOsm reabsorbed a much smaller absolute volume of water than did tap water animals there was a slight increase in the fractional reabsorption of the filtered volume (Table 9). The data of Kirschner et al. (1971) indicates that A. tigrinum larvae adapted for several days to Ringer's solution display a fractional reabsorption of water much higher than that of pond water adapted animals.

The tubular reabsorption of sodium and chloride in A. gracile is quite interesting. Tap water animals have the ability to reabsorb over 90 percent of the filtered sodium chloride while animals subjected to 200 mOsm reabsorbed only 84 percent of the sodium and

Table 9. Renal tubular handling of various substances under normal and reduced osmotic loads in A. gracile larvae. Values are mean ± SE (N). Fractional reabsorption (%R) is equal to (amount reabsorbed/amount filtered) x 100.

Substance	Tap water		200 mOsm		Significance of difference (%R)
	Amount reabsorbed or secreted (-)	%R	Amount reabsorbed or secreted (-)	%R	
Water (ml/10g-hr.)	0.026 ± 0.004 (19)	25	0.012 ± 0.002 (16)	39	*
Sodium (μeq/10g-hr.)	9.2 ± 0.7 (18)	92	2.5 ± 0.4 (15)	84	**
Potassium (μeq/10g-hr.)	0.7 ± 0.1 (17)	71	0.1 ± 0.0 (15)	57	**
Chloride (μeq/10g-hr.)	7.0 ± 0.6 (15)	94	2.1 ± 0.3 (15)	86	**
Urea (μAtm N/10g-hr.)	0.03 ± 0.03 (13)	40	0.01 ± 0.00 (5)	36	
Ammonia[1/] (μAtm N/10g-hr.)	-0.12		-0.05		

* Significantly different ($P < 0.05$)

** Significantly different ($P < 0.01$)

[1/] Calculated using a value of 0.44 μAtm N/ml for plasma ammonia-N concentration (Fanelli and Goldstein, 1964).

86 percent of the chloride (Table 9). A similar situation exists for potassium. Tap water animals reabsorb 71 percent of this ion while animals subjected to a reduced osmotic load reabsorb only 57 percent.

Plots of the absolute and fractional reabsorptions of sodium and chloride versus GFR are given in Figures 10 and 11. As GFR decreases the absolute reabsorption rate of sodium chloride decreases linearly while the fractional reabsorption rate remains almost constant, decreasing significantly in the lower GFR range (200 mOsm group).

The tubular handling of urea indicates a slight reabsorption for this compound in the kidney tubules of A. gracile (Table 9) but the magnitude was quite small. Tubular ammonia transfers were calculated using the value of 0.44 μAtm N/ml for Necturus maculosus for plasma ammonia - nitrogen (Fanelli and Goldstein, 1964). This was necessitated by the very low plasma ammonia concentrations found in these animals. The volume of plasma available is not sufficient for ammonia assay and the other analyses performed in this study.

The net transfer of ammonia, as would be expected, was into the tubule lumen in these experiments. Walker (1940) has determined that ammonia appears in the filtrate about one third of the way down the distal tubule and becomes more concentrated as the

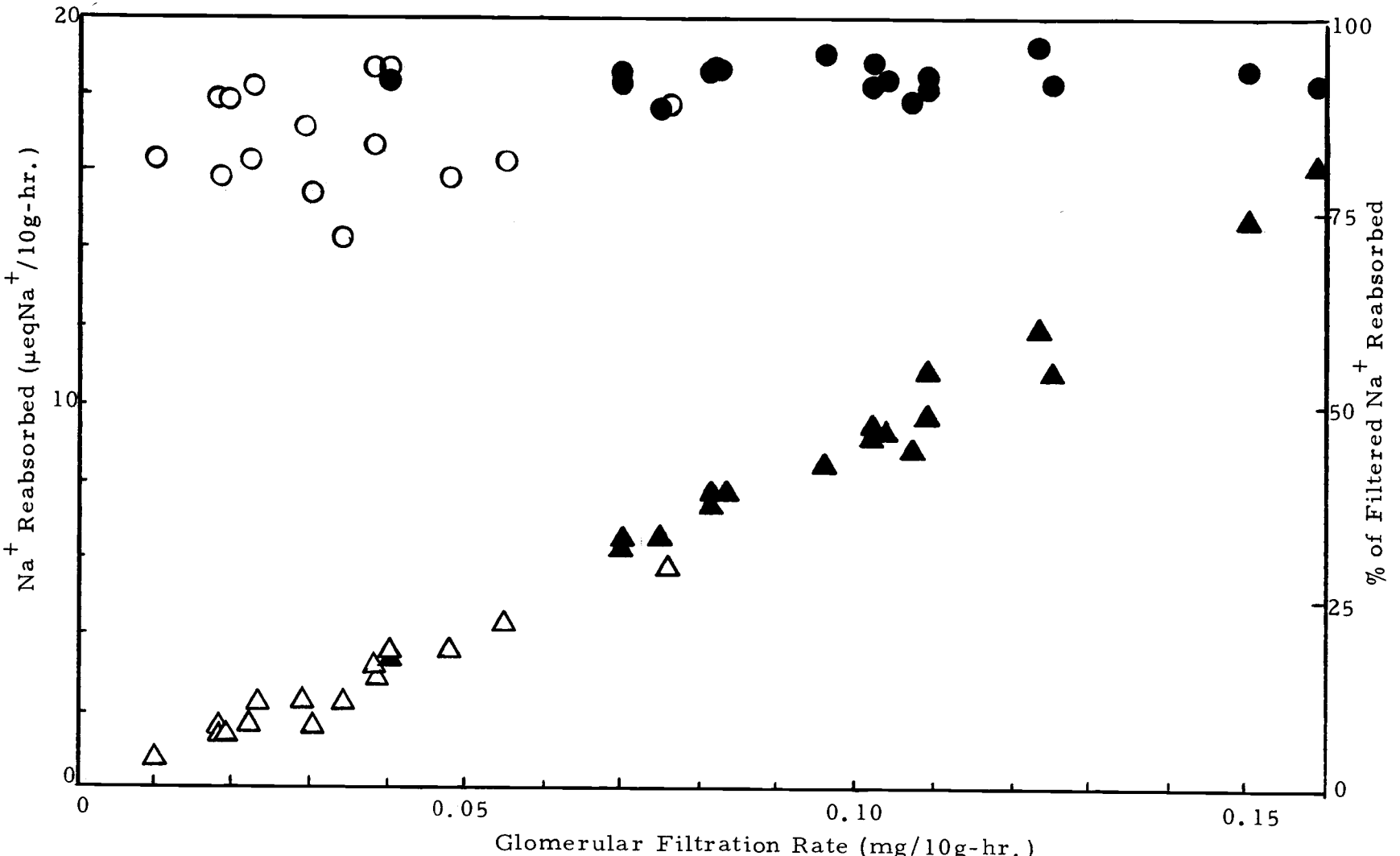

Figure 10. Absolute and fractional reabsorption of sodium as a function of GFR in A. gracile. Open figures are 200 mOsm animals. Closed figures are tap water animals. Circles are fractional reabsorption. Triangles are absolute reabsorption. Each point represents 1 animal.

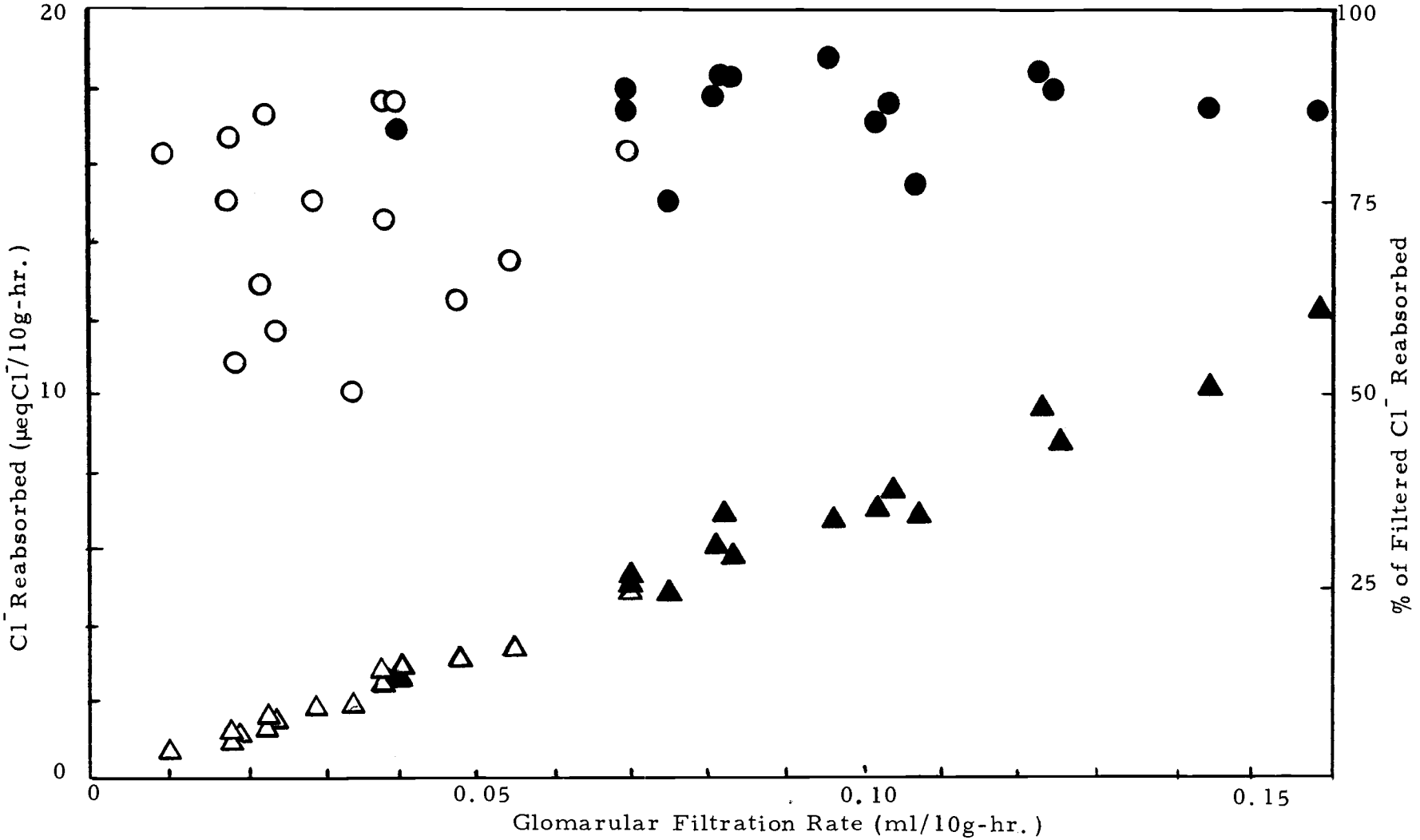

Figure 11. Absolute and fractional reabsorption of chloride as a function of GFR in A. gracile. Open figures are 200 mOsm animals. Closed figures are tap water animals. Circles refer to fractional reabsorption. Triangles refer to absolute reabsorption. Each point represents one animal.

Table 10. Renal tubular handling of phosphate in Ambystoma gracile in tap water.

		μAtm P/10g-hr.			
Animal	U/P (PO_4)	Filtered	Excreted	Reabsorbed	"Secreted"
1	0.61	0.31	0.16	0.15	
2	0.78	0.35	0.19	0.16	
3	0.97	0.43	0.36	0.07	
4	0.56	0.37	0.17	0.20	
5	1.18	0.22	0.13	0.09	
6	1.74	0.19	0.22		0.02
7	2.18	0.07	0.14		0.07
8	1.82	0.18	0.20		0.02
9	1.04	0.18	0.09	0.09	
10	1.35	0.25	0.21	0.04	
11	1.80	0.14	0.15		0.02
12	1.23	0.22	0.19	0.04	
$\bar{X}$	1.27	0.24	0.18	0.10	0.03
SE	0.15	0.03	0.02	0.02	0.01

fluid passes on through the collecting tubules.

The renal tubular handling of phosphate was quite variable (Table 10). About one third of the animals examined showed an apparently significant fractional reabsorption of phosphate having phosphate U/P values less than one. The remaining animals showed no significant net transfer of phosphate in either direction.

Walker and Hudson (1937b) noted reabsorption of phosphate in a few N. maculosus. The majority of the animals, however, merely concentrated the phosphate filtered without significant reabsorption or secretion.

Sodium Efflux in Free-Swimming A. gracile Larvae

The results of sodium efflux determinations with larvae whose cloacae were either ligatured closed or sham stitched are given in Table 11. It should be noted that the sodium efflux was lower prior to the stitching of the cloacae. The pre-stitched effluxes were 1.0 ± 0.1 (10) μeq/10g-hr. in tap water animals and 0.9 ± 0.1 (13) μeq/10g-hr. in 200 mOsm sucrose animals. The slight natriuresis which occurred after the stitching is probably attributable to the stress induced by the technique. Some bleeding may have occurred also.

The sodium effluxes determined here for tap water animals are in general agreement with previous determinations using this

Table 11. Sodium efflux in A. gracile under conditions of normal and reduced osmotic load. Values are mean ± SE (N). The animals weighed 9.6 ± 0.4 g (mean ± SE).

Component of efflux (μeq/10g-hr)	Tap water	200 mOsm
Total (sham stitched)	1.5 ± 0.1 (6)	1.4 ± 0.1 (6)
Extra renal (Ligatured cloacae)	1.0 ± 0.2 (6)	1.1 ± 0.1 (7)
Renal[a/]	0.5	0.3

[a/] Renal efflux = Total efflux - Extra renal efflux.

technique. Alvarado and Kirschner (1963), working with A. tigrinum, found sodium effluxes of 0.7 and 1.4 μeq/10g-hr. in stitched and sham stitched animals respectively. Parsons and Alvarado (1968) measured sodium effluxes of 0.8 and 2.0 μeq/10g-hr. in stitched and unstitched A. gracile larvae. The renal efflux of sodium in animals subjected to 200 mOsm sucrose was about half of that in tap water animals (Table 11). If the urine concentration of sodium remained the same after subjection to isosmotic sucrose one would expect a much greater decrease in renal efflux of sodium. This would be due to the over four-fold reduction in urine production seen in animals in 200 mOsm sucrose. Using the renal sodium efflux data obtained here and the urine production rates for A. gracile under the two conditions (Table 4), urine sodium concentrations can be approximated. When this is done the result is a sodium concentration of 6.5 μeq/ml for tap water animals and 16.5 μeq/ml for animals in 200 mOsm sucrose. The increase in urinary sodium concentration seen indirectly here in 200 mOsm sucrose animals is consistent with that seen in animals whose urine was collected for analysis (Table 6).

Effect of Long Term Exposure to Isosmotic Sucrose Solution on Na^+, K^+-ATPase Activity

Two groups of D. ensatus larvae were selected randomly and placed in individual containers filled with either tap water or 200

mOsm sucrose. The animals were weighed daily over a thirteen day period. The results, expressed as percent initial weight, are given in Figure 12. It can be seen that both groups lost weight rather rapidly over the first two or three days and then settled down into a much slower pattern of weight loss. This initial rapid weight loss probably results from a diuresis associated with the stress induced by daily weighing. If one compares the rates of weight loss in the two groups it can be seen that although the sucrose group appeared to lose weight more rapidly during the initial period, the difference was not significant. After the first two to three days the weight loss in the two groups became equal (0.4 percent/day).

At the end of the thirteen days the two groups were sacrificed and their kidneys removed. The kidney tissue was then homogenized and assayed for Na^+, K^+-ATPase. The Na^+, K^+-ATPase activity of the kidneys of the tap water group agreed quite well with activities of this enzyme in the kidneys of Rana catesbeinana (Asano et al., 1970). These workers found activities of 10.2 and 43.3 μmoles Pi/mg protein-hr. at 22°C and 37°C respectively. The results indicate that the activity of this enzyme decreases in D. ensatus after prolonged exposure to elevated external concentrations. The tap water group showed Na^+, K^+-ATPase activity of 20.0 ± 6.1 (7) μmoles Pi/mg Protein-hr. while the 200 mOsm sucrose group revealed an activity of 8.3 ± 2.0 (7) μmoles Pi/mg protein-hr.

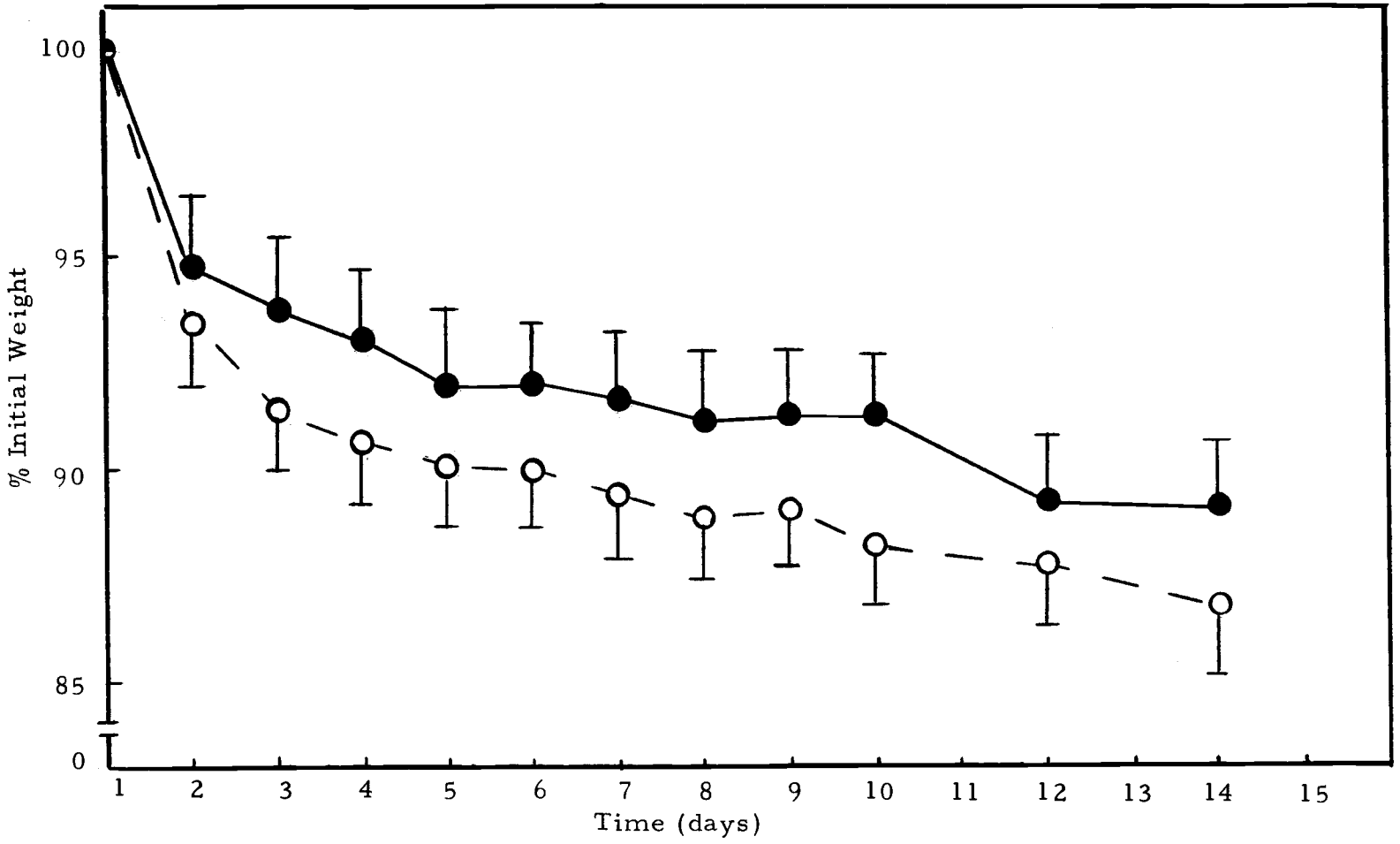

Figure 12. Weight loss as a function of time. Each point is mean and standard error. Open circles are animals in 200 mOsm sucrose (N = 7). Closed circles are animals in tap water (N = 7). Species is D. ensatus.

However, the reduction is only marginally significant ($P < 0.10$, two sided t-test). Although there was over a three-fold reduction in ATPase activity in the 200 mOsm group, there was a very high degree of variability in the tap water group. It is probable that given a larger N the difference would become significant. If this is true it would provide a correlation at the biochemical level with the observation that the fractional reabsorption of sodium decreases when the animals are exposed to reductions in osmotic load (Table 9).

DISCUSSION

The vertebrate kidney plays a major role in maintaining homeostasis. Through the processes of filtration of plasma, reabsorption of water and solutes from the filtrate and secretion of waste products into the filtrate, this organ succeeds in ridding the organism of toxic waste products while conserving materials necessary to life. The kidney thus functions in excretion of waste products, osmotic regulation and solute conservation. The relative importance of these three functions varies with the habitats of different animals. Terrestrial forms, such as most mammals, are faced with the dual problem of elimination of waste products and the concentration of water. These animals have kidneys which have evolved mechanisms for the production of a highly concentrated urine. This enables the animals to conserve water while excreting significant quantities of waste products such as urea.

Animals such as fresh water fish and amphibians are faced with a quite different problem. In the case of aquatic amphibians the renal excretion of nitrogenous waste products is not nearly as important as it is in mammals. Aquatic amphibians lose large quantities of waste nigrogen across their skin in the form of ammonia. The kidneys in these forms function primarily to excrete large

volumes of osmotically obtained water and secondarily, to conserve solutes.

The balance between osmotic uptake of water and urine production must be very precise. External factors such as temperature and osmotic load effect the rate of osmotic uptake of water greatly. The animals must be able to perceive changes in these environmental factors and adjust their urine production accordingly. Failure to do either would result in either dehydration or over-dilution of the body fluids.

The fundamental unit of the vertebrate kidney is the glomerulus and its associated nephric tubule. Figure 13 is a diagramatic representation of this complex as found in a typical amphibian. The blood enters the glomerular tuft of capillaries through the afferent arteriole. In the glomerulus some of the plasma is filtered as a result of the hydrostatic pressure within the glomerulus. The remaining fluid leaves the glomerulus via the efferent arteriole. This arteriole branches into a series of peritubular capillaries which pass in close association with the nephric tubule. Products reabsorbed from the tubule are returned to the circulation at this point. These capillaries ultimately converge into the renal veins which then leave the kidney.

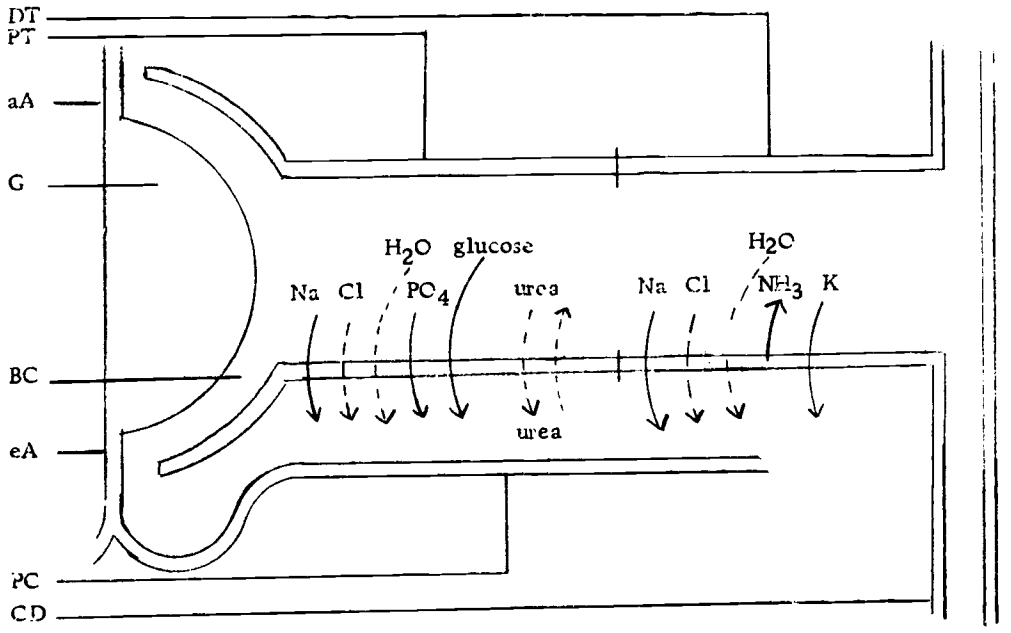

Figure 13. Diagram of a typical amphibian glomerulus and its associated nephron. Solid arrows indicate direction of active transport. Broken arrows indicate direction of passive movement. Bowman's capsule (BC); Collecting ducts (CD); Afferent Arteriole (aA); Efferent Arteriole (eA); Glomerulus (G); Peritubular capillary (PC); Proximal Tubule (PT); Distal Tubule (DT).

Subsequent to filtration the fluid finds its way into the nephric tubule via Bowman's capsule. In the first portion of the tubule, the proximal segment, such solutes as glucose, phosphate and sodium are reabsorbed from the filtrate by active transport. Chloride follows the electrical gradient set up by sodium reabsorption (lumen is electronegative to blood). Some of the water in the tubule is also reabsorbed here as a result of the osmotic gradient generated by sodium reabsorption. As a result of these processes the volume of

the filtrate decreases in the proximal tubule. Concurrently, the composition of the filtrate changes, particularly with respect to glucose. As most of the filtered glucose is removed in the proximal segment there is little, if any, glucose left in the fluid that reaches the distal segment. Other solutes, such as potassium, urea, and possibly phosphate which are reabsorbed only slightly or not at all in this segment, become more concentrated as the fluid enters the distal tubule. Still others, such as sodium and chloride which are reabsorbed in an isosmotic solution with water remain unchanged in their concentration until they enter the distal tubule.

Once the fluid enters the distal tubule it is changed further. Although still more water is reabsorbed here, the reabsorption of sodium and chloride occurs in a hyperosmotic fluid so that the fluid left behind in the tubule is diluted with respect to these ions. Potassium ion is also reabsorbed in this segment. With the removal of water from the tubular fluid, the concentration of urea has risen all along the tubule. When the concentration of this product exceeds the concentration in the plasma in the peritubular circulation urea diffuses passively back into the blood. Ammonia makes its first appearance in the fluid of the distal tubule. This nitrogenous waste product is secreted into tubular fluid and originates from a process of deamination of amino acids within the tubule cells (Balinsky and Baldwin, 1962).

When the fluid leaves the distal tubule it enters collecting ducts. In these structures the fluid is transported via the ureters to the cloaca. Once in the cloaca, the urine may enter a urinary bladder for further modification by selective reabsorption of water and important solutes. This is especially true in adult amphibians which inhabit terrestrial habitats where water retention is critical. Urine which is not stored in the bladder is voided from the organism through the cloaca.

The Question of the Role of the Bladder in Urine Formation

Although a great deal is known of the role played by the anuran bladder in the process of urine formation, a similar body of data concerning the urodelian bladder has not accumulated (see review by Bentley, 1966).

Of the studies undertaken to determine the function of the salamander bladder most have been concerned with the adult form of the animal. It has been demonstrated that both isolated and *in vivo* bladders of adult *Salamandra maculosa* reabsorb considerable volumes of water from the urine they contain. Furthermore, the administration of arginine vasotocin (AVT), a posterior pituitary antidiuretic hormone, greatly enhances this reabsorption (Bentley and Heller, 1965). Alvarado (1972a) has demonstrated appreciable reabsorption of water from the bladders of dehydrated *A. tigrinum*

adults although sodium transport was not apparent.

Investigations concerning the role of the bladder in larval urodeles have been limited. Although slight water movement across isolated bladders of larval Ambystoma mexicanum and N. maculosus has been shown by Bentley and Heller (1964) it was not affected by AVT. Adult A. mexicanum adapted to an aquatic environment gave indications of sodium reabsorption from bladder urine (Aceves et al., 1970).

The results reported here for larval A. gracile bladders indicated that very little if any water is reabsorbed when urine is forced to remain in the bladder (Figure 2). More important, however, is the fact that the animals appeared to urinate more or less continuously, with very little bladder retention of urine (Figure 1a).

Renal Function in Urodeles

The high degree of correlation shown between glomerular filtration rates in larval A. gracile with and without urine collection bags and the agreement of these values with GFRs determined by other workers (Kirschner et al., 1971) on closely related species indicates that the urine collection bag technique is capable of giving reliable results. Further evidence for this comes from urine concentrations of the several substances assayed. Allowing for a certain predictable degree of saluresis (owing to the stress induced

by the bags) the concentrations of most urine solutes agreed quite well with previously published values.

One of the more interesting, and perhaps most dramatic, results of this study was the facility with which glomerular filtration rate can be adjusted over a wide range by simply altering the osmotic load presented to the animals (Figure 4). The advantage of this is that it makes it possible to study the effect of changes in filtration rate on a variety of processes without the need for such surgical procedures as renal artery clamping. Furthermore, since only the total solute concentration is altered in the bathing medium, the animals were exposed to more or less normal ionic concentrations.

Decreases in GFR have been noted in amphibians under a variety of conditions. Examples are dehydration (Schmidt-Nielson and Forster, 1954), low temperature (Parsons and Alvarado, 1968) and reduced osmotic load due to increased external solute concentration (Schmidt-Nielson and Lee, 1962; McBean and Goldstein, 1970; Kirschner et al., 1971). Naturally the mechanism of this decrease in GFR is of great interest.

It was observed long ago that injection of posterior pituitary extracts causes a transitory weight gain in larval and adult Ambystoma tigrinum (Belehradek and Huxley, 1927). A renal effect was postulated in the response. It has also been long known that these extracts can cause vasoconstriction in the afferent glomerular

arterioles of the frog (Richards and Schmidt, 1924). Constriction at this point would decrease blood flow into the glomerulus and thus slow GFR. In later investigations (Sawyer, 1951, 1957) it was demonstrated that injection of posterior pituitary extract does indeed cause a decrease in GFR of toads. The response to the active principle was later shown to be identical to the response to AVT (Uranga and Sawyer, 1960). The effect of AVT injection on GFR in urodeles has also been investigated. It has been demonstrated that this hormone causes a decrease in glomerular filtration in larval A. tigrinum (Alvarado and Johnson, 1965). The relationship between the effects of AVT and reduced osmotic load on GFR remains unclear, however. It has been shown that Rana catesbeiana adults adapted to 150 mM sodium chloride appear to have more neurosecretory material in their preoptic nuclei than do tap water adapted frogs (Ridley, 1964). On the other hand hypophysectomy and lesioning of the preoptic nuclei failed to abolish the decrease in GFR exhibited by larval A. tigrinum adapted to Ringer's solution (Kerstetter and Kirschner, 1971).

Another possible mechanism for the observed decrease in GFR is the renin-angiotensin system. The final product of this hormonal system, angiontensin II, has marked vasoconstrictor activity and could, if specific for the afferent glomerular arteriole, cause a reduction in GFR. Recent investigation, however, places the renal

vasoconstrictor effect of angiotensin II at the efferent glomerular arteriole in the bullfrog (Sokabe et al., 1972). This would imply a situation in reverse of the control of filtration hypothesized for AVT. In the case of the renin-angiotensin system the hormone would function to increase filtration rate by increasing the filtration fraction. Indeed higher plasma renin activities have been found in hydrated bullfrogs than in those which had been dehydrated or subjected to reductions in osmotic load (Sokabe et al., 1972). Hydrated amphibians of course maintain higher filtration rates than do dehydrated animals.

Complicating the renin-angiotensin hypothesis is the widely held belief that amphibians (Forster, 1942) and quite possibly mammals (Handley and Moyer, 1955; Van Liew, Deetjen and Boylan, 1967; Keys and Swanson, 1971) decrease their GFR by decreasing the number of active glomeruli. This was also observed here for D. ensutus (Figure 8). Of course such glomerular intermittency is completely compatable with afferent arteriolar constriction. However, efferent arteriolar constriction could not interrupt filtration in the glomerulus. It would seem it is quite possible that both mechanisms are operable in regulating GFR; the renin-angiotensin system could increase filtration during conditions of hydration and AVT could decrease GFR when the animals became dehydrated.

Such a mechanism would confer great sensitivity to the control of filtration rate.

The latent period between the increase in external osmotic concentration and the response of reduction of GFR has not been extensively investigated. McBean and Goldstein (1970) report that X. laevis decreases GFR within a few hours of contact with concentrated saline. The finding here is that D. ensatus reduces filtration rate between two and three hours after the elevation of the concentration of the external medium (Figure 6). Ambystoma gracile appears to respond in a similar manner. The return to approximately normal GFR following replacement of 200 mOsm sucrose with tap water appeared to require a greater amount of time than did the initial decrease (Figure 7). This might be expected if the response is hormonally mediated. One cannot assume that the time required for perception of a change in osmotic load and the initiation of the physiological response to that change requires the same amount of time whether the change is an increase or a decrease. If the release of hormone is the response to a decreased osmotic load (as is implied here) then the response to a change back to a normal osmotic load would require the time needed for perception of the change, the time needed to reduce the release of the hormone and, finally, the time required for the hormone to be eliminated from the circulation.

Quite possibly this latency would be greater than the latency involved in the initial response.

The fact that these animals are able to adjust their urinary output in response to changes in osmotic load implies an ability to recognize those changes. This recognition could either be direct with external osmoreceptors or indirect with internal osmoreceptors which respond to changes in body fluid concentration. The latter system is present in mammals and might well be present in amphibians although it is yet to be described. Organs which might be external osmoreceptors have been reported in amphibians. Andersson and Zotterman (1950) described sensory receptors in the tongue of Rana temporaria and R. esculenta which respond to water and dilute sodium chloride solutions. These receptors might be involved in monitoring osmotic load in these animals.

Comparison of the tubular handling of water and dissolved solutes in intact kidneys of A. gracile larvae with previous data obtained by the micropuncture technique is quite interesting. The agreement between the results of the two methods was quite good although exceptions did exist. It was found here that A. gracile larvae, adapted to tap water, reabsorbed approximately 25 percent of the water filtered at the glomeruli (Table 9). This is lower than usually accepted values obtained by mircopuncture. It has been estimated by using "free-flow" micropuncture techniques that from

20 percent (Walker and Hudson, 1937a) to 50 percent (Bott, 1962) of the water filtered by Necturus is reabsorbed in the proximal tubule alone. Similarly, it has been reported that Amphiuma reabsorbs 25-30 percent of the filtered water in its proximal tubules (Wiederholt, Sullivan and Giebisch, 1971). Approximately 50 percent of the reabsorbed water leaves the tubule in the distal segment of amphibian nephrons (Bott, 1962; Wiederholt, Sullivan and Giebisch, 1971). This would imply that up to one half of the filtrate is reabsorbed in these animals. Fractional reabsorption of water can be calculated from a knowledge of inulin U/P values. Reported U/P values for intact urodeles in pond water include 1.1 (Kirschner et al., 1971). 2.5 (Alvarado and Johnson, 1965) and 3.3 (Parsons and Alvarado, 1968). The values reported here are 1.4 for A. gracile and 1.4 for D. ensatus in tap water. This range in U/P values corresponds to a range of 10-70 percent reabsorption of filtered water. The data obtained by both techniques (micropuncture and inulin U/P values) indicates that there is a great deal of variability in fractional water reabsorption within this group.

It is interesting to note that several A. gracile U/P values were quite close to one and two were below unity. Inulin U/P values below one have also been noted by Alvarado (1972b) in A. gracile Inulin U/P values of close to (or less than) one imply either that the tubular epithelium has a very low permeability to water or

that water is moved into the tubule lumen.

Measurements of tubular reabsorption of sodium reported here for A. gracile averaged 92 percent of filtered load (Table 9). This is in very close agreement with previously reported data. Bentzel, Anagnostopoulos and Pandit (1970) have determined that 95 percent of the filtered sodium is reabsorbed renally in intact, anesthetized Necturus. Wiederholt, Sullivan and Giebisch (1971) report 90 percent reabsorption of sodium in Amphiuma using "free-flow" micropuncture techniques.

The fractional reabsorption of chloride ion in A. gracile in tap water was quite similar to that of sodium. It was found that 94 percent of the filtered chloride was reabsorbed in these animals (Table 9).

The tubular handling of chloride ion has not received nearly as much attention as has sodium. Bott (1962) reports a decrease in tubular fluid chloride concentration from 81 μeq/ml in the beginning of the proximal tubule to 4 μeq/ml in the collecting ducts of Necturus. Since these values were taken from individual tubules in different animals it is not possible to calculate the fractional reabsorption. However, the drop in concentration is consistent with what one might expect if about 94 percent of the chloride was reabsorbed.

The fractional reabsorption of potassium ion was lower than

that of sodium and chloride. It was observed that about 71 percent of the filtered potassium was reabsorbed by the kidneys of A. gracile in tap water. This agrees quite well with the value obtained by Wiederholt, Sullivan and Giebisch (1971) of 75 percent reabsorption of potassium in Amphiuma.

The tubular handling of phosphate reported here for A. gracile agrees quite well with the data previously published (Table 10). Net reabsorption was seen in about half of the animals examined while the other half failed to display a significant difference between the quantity filtered and the quantity excreted. Walker and Hudson (1937b), working with Necturus, found that although reabsorption of this ion was occasionally seen in proximal tubules it was usually concentrated in the tubules by the removal of water. They argued that sufficient water was reabsorbed from the tubules to preclude the necessity of invoking secretion as the mechanism of the elevation of phosphate concentration in the distal segment. Subsequently, Hogben and Bollman (1951) demonstrated marked tubular reabsorption of phosphate in the frog kidney and provided sound evidence against its secretion. They showed that $^{32}PO_4$ introduced into the renal portal vein did not appear in significant quantities in the urine. Since the renal portal vein supplies only peritubular capillaries and not glomeruli, this is taken as positive proof that phosphate is not secreted into the tubular lumen.

The fact that a few of the animals investigated here quite actively reabsorbed phosphate may be related to their metamorphic stage. A great deal of phosphorous is required for osteogenesis, a process very much in evidence during metamorphosis. As these animals were collected and investigated in the spring when larval forms of this species undergo metamorphosis (Stebbins, 1954) it is likely that they were in different stages of transformation.

A net reabsorption of 40 percent of the filtered urea was observed in A. gracile in this study. The virtual identity of urine and plasma urea concentrations (Table 6) reported here for A. gracile indicates that a passive equilibrium exists for urea between the tubular fluid and plasma. This would mean that the reabsorbate remains in equilibrium across the tubules and would indicate a high degree of permeability to urea in the tubular epithelium. It has been shown that the proximal tubule of Necturus is permeable to urea (Walker and Hudson, 1937a). It has further been shown that there is considerable passive reabsorption of urea from the tubular lumen into the peritubular capillaries in the bullfrog (Love and Lifson, 1958). This net movement presumably occurs as a result of the high permeability of the tubular epithelium to urea, the concentration gradient set up by reabsorption of water and, in the case of the frog, tubular secretion of urea (Forster, 1954).

Renal handling of nitrogenous waste products among the

amphibia has received considerable attention. This is especially true with respect to the secretion of urea by some anurans. It has been demonstrated that secretion of urea from the peritubular capillaries into the nephric tubule occurs in several species of frogs (Marshall and Crane, 1924; Walker and Hudson, 1937a; Forster, 1954; Carlisky et al., 1970). Similar secretion of urea has not, however, been demonstrated in Necturus, the only urodele previously investigated in this respect (Walker and Hudson, 1937a).

The excretion of ammonia, which is the major waste product of nitrogen metabolism in A. gracile (Table 9) probably occurs primarily across the skin. Although only 57 percent of the urinary component of total nitrogen excretion is in the form of ammonia most of this product is lost extra renally. Alvarado and Dietz (1970b) report total ammonia excretion of 2.5 μAtm N/10g-hr. in A. gracile. The urinary excretion rate of ammonia seen here ($\dot{V} \times U_{NH_3}$, Tables 4 and 6) is 0.17 μAtm N/10g-hr. Thus, over 90 percent of the excreted ammonia leaves the animal by extra renal routes. A similar situation has been reported by Fanelli and Goldstein (1964) for Necturus. In the present study ammonia is secreted by the kidneys of A. gracile. Of course the plasma value of 0.44 μAtm NH_3-N/ml (Fanelli and Goldstein, 1964) used for these calculations probably introduces some error but since the amount excreted is almost four times the probable amount filtered, ammonia

must have been added to the tubule subsequent to filtration. It has been shown (Walker, 1940) that the transfer of ammonia into the kidney tubule occurs in the distal segment of Necturus and cannot be explained by secretion of the substance across the tubule wall into the lumen because perfusion of the peritubular circulation with ammonia-free solutions failed to abolish the addition of NH_3 into the tubular fluid. It was suggested by Walker (1940) that deamination of some precurser occurs in the cells of the distal tubules and that ammonia is then transferred into the lumen. Direct evidence for the deamination of several amino acids, especially alanine, in the tubular cells of Xenopus laevis have been supplied by Balinsky and Baldwin (1962).

The effect of a reduction in osmotic load on the tubular handling of water and solutes in the filtrate was quite interesting. The slight increases in inulin U/P values (Tables 4 and 5) and fractional reabsorption of water (Table 9) in the animals subjected to 200 mOsm sucrose may indicate an increased permeability of the tubular epithelium to water. Kirschner et al. (1971) and Kerstetter and Kirschner (1971) have reported greater increases in inulin U/P values in Ambystoma tigrinum adapted to Ringer's solution for several days. It is quite possible that the onset of the tubular response(s) may require a much longer period of exposure to reduced osmotic load than does the GFR response. If this is the case the reduction in

GFR might serve as an initial but incomplete adjustment to the decreased osmotic load which would later be followed by the final adjustment of increased fractional water reabsorption. The pattern of weight loss of animals in 200 mOsm sucrose indicates that this might be the case (Figure 12). It can be seen that although the 200 mOsm sucrose group appeared to lose weight slightly faster than the control group over the first two or three days they did survive and eventually settled into a pattern of weight loss identical to that of the control group.

The mechanism of the increased fractional reabsorption of water may involve arginine vasotocin. Increases in fractional tubular reabsorption of water after injection of posterior pituitary extracts (Sawyer, 1957) or AVT (Uranga and Sawyer, 1960) have been reported in toads. A relationship between the responses to injected AVT and reduced osmotic load may exist in urodeles. Hypophysectomy and lesioning of the preoptic nuclei in A. tigrinum larvae is known to prevent increases in inulin U/P values in animals adapted to Ringer's solution (Kerstetter and Kirschner, 1971). The ability of the tubular epithelium to reabsorb greater fractions of filtered water after treatment with AVT is usually attributed to an increased permeability to water. Whittembury, Sugino and Solomon (1960) have demonstrated an increase in equivalent pore radius in proximal tubule epithelium in kidney slices of Necturus treated with

mammalian posterior pituitary extract.

In 200 mOsm sucrose the urine concentration increased with respect to each substance assayed in both species. Since the animals were only exposed to 200 mOsm sucrose for four hours before the urine collection began, these are taken to be acute responses. Sodium, potassium and chloride concentrations in the urine of A. gracile exposed to 200 mOsm sucrose increased by roughly the same factor over the concentrations of these ions in the urine of tap water animals. The close agreement in degree of increased concentration over the urine of animals in tap water did not occur in D. ensatus exposed to 200 mOsm sucrose. This may be a result of the small number of animals examined.

In the case of A. gracile urine, increased sodium concentration in 200 mOsm animals was seen under two sets of experimental conditions, direct analysis of collected urine (Table 6) and calculation from the knowledge of renal efflux of sodium and $\dot{V}$. Under each of these conditions the urine of 200 mOsm animals was two-three times as concentrated with respect to sodium. The same ratio of two-three held for urine chloride when urine was collected and analysed directly. Potassium concentration increased by a factor of 1.7 in the 200 mOsm sucrose group.

It has been reported that the urine of frogs (Gordon, 1962; Mayer, 1969) and the salamander A. tigrinum (Kirschner et al., 1971)

increases in sodium concentration when these animals are adapted to concentrated saline. The data presented here indicates that increases in urine sodium as well as potassium and chloride concentrations occur when salamanders are acutely exposed to increases in osmotic concentration without concomitant increases in ionic concentration.

Much of the increased urinary concentration in these ions can be explained by the observed increase in the fractional reabsorption of water seen in the 200 mOsm group. However, this concentrating effect will not account for all of the increase as the fractional reabsorption of several ions also decreases when animals are exposed to 200 mOsm sucrose. The fractional reabsorption of sodium in these animals was 84 percent. Fractional reabsorption of chloride was 86 percent while it was 57 percent for potassium in A. gracile in 200 mOsm sucrose. In each case the decrease below tap water values was significant ($P < 0.05$). It is known from isotopic tracer studies that there is a large diffusive backflux of sodium and potassium from the peritubular capillaries in the bullfrog (Hoshiko, Swanson and Visscher, 1956). A similar backflux has also been demonstrated in Necturus (Oken et al., 1963). It is possible that an increased backflux in the 200 mOsm sucrose group could explain the observed decrease in fractional reabsorption, particularly if there is an AVT-induced increase in tubular permeability.

Although a decrease in the active component of the net reabsorptive transfer might be indicated by the possible decrease in the activity of Na^+, K^+-ATPase in the kidneys of animals chronically exposed to 200 mOsm sucrose, it seems doubtful that such changes could occur under acute conditions.

In view of the rather large renal excretion of sodium in the 200 mOsm sucrose group it might be expected that these animals suffer rather severe net losses of this ion. This is not necessarily the case. It has been shown that the hormone aldosterone enhances influx of sodium but has no effect on the efflux of the ion in larval A. tigrinum (Alvarado and Kirschner, 1964). The authors interpreted this to mean that the hormone stimulates transport of sodium in the skin but has no effect on renal tubular sodium reabsorption. The release of such a hormone would obviously be of value to animals subjected to decreases in osmotic load.

If arginine vasotocin is involved in the increased fractional reabsorption of water in A. gracile in 200 mOsm sucrose its role in the renal sodium transport of these animals remains unclear. It has been reported that AVT stimulates sodium transport in the renal tubules of several higher vertebrates including the frog (Jard and Morel, 1963), a water snake (Dantzler, 1967) and the domestic chicken (Ames, Steven and Skadhauge, 1971). Furthermore, the mammalian antidiuretic hormone arginine vasopressin stimulates

sodium transport in rabbit renal collecting tubules (Frindt and Burg, 1972). Alvarado and Johnson (1965) noted a reduction of GFR but no change in urinary sodium concentration in larval A. tigrinum injected with AVT. Similar constancy of urinary sodium concentration after injection of AVT into the urodeles Triturus alpestris, T. cristatus and Necturus maculosus has been reported by Bentley and Heller (1964). Tubular effects of the hormone were not evaluated in either study, however. The finding here is that exposure to 200 mOsm sucrose causes a slight increase in fractional water reabsorption and a decrease in fractional sodium reabsorption in urodeles. It would be interesting to evaluate the effect of AVT on renal sodium reabsorption in these animals.

If the rates of reabsorption of sodium (Figure 10)or chloride (Figure 11) are plotted as a function of glomerular filtration rate it is seen that there is a linear decrease in the rate of absolute reabsorption while fractional reabsorption remains constant in the tap water group. A rather close agreement between the reabsorption rates of sodium and chloride is seen here for the whole kidney. It is known that chloride is passively reabsorbed in Necturus proximal tubules (Giebisch and Windhager, 1963, 1964), following the electrical gradient set up by the active reabsorption of sodium out of the tubule. Further evidence for the passive reabsorption of chloride is supplied by Eigler (1961) who showed that substitution of

sulfate ion for chloride in the perfusate of proximal tubules causes an increase in potential difference but no change in short circuit current across the tubule wall. The tubular epithelium is much less permeable to sulfate than to chloride. Since the potential difference is a function of the separation of charge between the actively transported sodium cation and the anion which follows passively, the substitution of chloride with sulfate would result in a greater separation of charge and, thus, a greater potential difference. Chloride is also reabsorbed across the distal tubule of Necturus (Bott, 1962) although the nature of this transport is unclear. Evidence for active chloride transport has been reported for mammalian distal tubules (Rector and Clapp, 1962).

The linear relationship between absolute rate of reabsorption of sodium chloride and GFR noted here for A. gracile (Figures 10 and 11) is a manifestation of glomerulotubular balance. This phenomenon has received little attention in amphibians. Because of the ease with which GFR can be varied in these animals by simply varying osmotic load, they appear ideally suited to the study of glomerulotubular balance.

The concept of glomerulotubular balance was first introduced by Smith (1951) to describe the ability of the kidney to alter the rate of solute reabsorption with alterations in filtration rate. If the absolute reabsorptive rate of any solute remained fixed during a

decrease in GFR the fractional reabsorptive rate would increase leading to an "over-conservation" of the solute. Conversely, an increase in GFR would lead to an increased excretion of the solute and a net loss to the animal. In recent years the term glomerulotubular balance has come to refer primarily to the regulation of sodium excretion during alterations in filtration rate and has been extensively studied in mammals (Glabman, Aynedjian and Bank, 1965; Rector, Brunner and Seldin, 1966; Levine et al., 1968; Brenner, Bennett and Berliner, 1968).

Although it has been amply demonstrated that vertebrates decrease GFR by decreasing glomerular activity it seems unlikely that this is accomplished by deactivating succeeding numbers of glomeruli while the others continue to filter at a constant rate. It has been suggested that, in the dog, the filtration rate in each individual glomerulus which remains active decreases as the total GFR decreases (Keys and Swanson, 1971). If this is the case in amphibians glomerulotubular balance would serve to regulate sodium excretion in those nephrons which remain functional at any given GFR.

The amphibian kidney occupies a unique place in the evolution of renal function. The ancestors of the amphibians, freshwater fishes, occupied an environment which subjected their tissues to continuous flooding as a result of osmotic uptake of water. To

combat this these fish evolved highly efficient kidneys for the removal of this water. The descendents of these fish, the amphibians, occupy the same aquatic habitat for at least part of their life. It is not surprising, therefore, that they should also have kidneys capable of forming large quantities of dilute urine.

Most freshwater fish are confined to an aquatic environment exclusively. Amphibians, on the other hand, venture out of the water for variable lengths of time. The maintenance of a copious urine production during these periods would, of course, lead to rapid dehydration. To prevent this, amphibians have developed regulatory systems to control their urine output. The major contribution to this control is provided by the endocrine system. Antidiuretic hormones such as arginine vasotocin decrease urine production by decreasing filtration rate and increasing the permeability of the renal tubules to water. It is not surprising that these control systems seem to be missing in fresh water fishes. For example, arginine vasotocin produces diuresis and natriuresis in the lungfish by increasing GFR, C_{H_2O} and C_{Na} (Sawyer, 1966).

The descendents of the amphibians have advanced on the water retaining mechanisms of their ancestors'kidneys. This is particularly true of the higher forms such as mammals which excrete small quantities of concentrated urine.

Because of the unique position in the phylogenetic scheme

occupied by amphibians, they are extremely interesting animals on which to study renal physiology. This is particularly true of the urodeles which are the most primitive group of amphibians readily accessable for study. The control of renal function in this group is only beginning to receive the attention it deserves.

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APPENDIX

APPENDIX

Detailed Protocols for the Chemical Analysis of Urine, Plasma and Bath Samples

1. Urea Analysis (Method of Coulombe and Favreau, 1963).

Reagents:

a) H_3PO_4-$FeCl_3$ solution.

100 mg $FeCl_3$ were dissolved in sufficient 85% (v/v) phosphoric acid to make one liter of solution.

b) Diacetyl monoxime-thiosemicarbazide solution.

2.5 g diacetyl monoxime and 0.12 g thiosemicarbazide were dissolved in sufficient distilled water to make 100 ml of solution. The solution was made fresh for each analysis.

Standard Solutions:

a) Stock Solution.

100.0 mg urea was dissolved in sufficient distilled water to make one liter of solution. This was stored frozen at -10°C.

b) Working Solution.

The stock solution was brought to room temperature and diluted 10:1 with distilled water before each analysis. 1 ml contains 10 μg urea.

Analysis of urine and plasma samples.

a) 10 or 20 μl of urine or plasma were added to 3 ml distilled water. A distilled water blank and a series of urea standards were prepared.

b) 2 ml of the H_3PO_4-$FeCl_3$ solution was added to each test tube.

c) 0.2 ml of the diacetylmonoxime-thiosemicarbazide solution was added to each tube and the tubes were mixed with a vortex mixer.

d) The tubes were capped with marbles and heated in boiling water for 20 minutes.

e) After heating the tubes were cooled in tap water immediately and then the optical density of each was read at 530 nm on a colorimeter against the blank.

f) Urea concentrations were read off a standard curve.

2. Inulin Analysis (Nakamura's modification for fructose, 1968, of the method of Roe et al., 1949).

Reagents:

a) Resorcinol-thiourea solution.

0.5 g resorcinol and 1.25 g thiourea were dissolved in sufficient glacial acetic acid to make 500 ml of solution. This was stored in a brown bottle under refrigeration.

b) Hydrochloric acid, 30%.

Five parts of concentrated HCl (Sp. Gr. = 1.19) were added to one part distilled water.

Inulin Standard Solution.

a) Stock Solution.

100.0 mg inulin was dissolved in sufficient distilled water to make 100 ml of solution.

b) Working solution.

The stock solution was diluted 50:1 to a final concentration of 20 μg inulin/ml.

c) The standard for the plasma analyses was made up separately and contained 1 mg/ml.

Note: The stock solution was made fresh for each series of analyses and stored in a refrigerator between analyses. The working solution was made up fresh before each analysis.

Analysis of bath samples.

a) 1 ml bath samples were taken and placed in test tubes setting in an ice bath.

b) After at least 5 minutes 3.5 ml 30% HCl was added to each tube. A distilled water blank and a series of standards were prepared in a similar manner.

c) 0.5 ml of the resorcinol-thiourea solution was added to each tube. The contents were mixed on a vortex mixer.

d) Each tube was heated for 25 minutes at 70°C.

e) After heating the tubes were cooled in tap water and the optical density at 510 nm was read against the blank in a colorimeter.

f) Inulin concentrations were read off a standard curve.

Analysis of plasma samples.

a) 50 µl of distilled water was pipeted into each of several microfuge tubes.

b) 20 µl of plasma, inulin standard (20 µg inulin) or distilled water were added to each.

c) 20 µl trichloroacetic acid was added to each and the precipitated protein was pulled down by centrifugation.

d) 50 µl of each supernatent were added to 3.5 ml of chilled HCl (30%) and the analysis proceded as above for bath samples.

3. Phosphate Analysis (method of Murphy and Riley, 1962).

Reagents:

a) Potassium antimonyl tartrate

1.3715 g of $K(SbO)C_4H_4O_6 \cdot 1/2\ H_2O$ were dissolved

in sufficient distilled water to make a 500 ml solution.

b) Ammonium molybdate solution.

20 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ was dissolved in sufficient distilled water to make 500 ml of solution. (Stored in a plastic bottle in a refrigerator.)

c) Ascorbic acid solution.

1.76 g of ascorbic acid was dissolved in sufficient distilled water to make 100 ml. This was made fresh within a week of use and stored in a refrigerator.

Standard phosphate solutions.

a) Stock solution.

0.1098 g of KH_2PO_4 was dissolved in sufficient distilled water to make 500 ml of solution.

b) Working solution.

The stock solution was diluted 100:1 with distilled water. The final concentration was 0.5 μg P/ml (all P derived from PO_4)

c) The stock standard was used for plasma analyses and contained 50 μg/ml of P.

Combined reagent.

The reagents were mixed in the following order with stirring after the addition of each.

1) 50 ml 5 N H_2SO_4; 2) 5 ml potassium antimonyl

tartrate; 3) 15 ml ammonium molybdate; 4) 30 ml ascorbic acid. The combined reagent was prepared fresh before each analysis.

Analysis of urine samples.

a) 10 or 20 µl of urine was added to 2.5 ml of distilled water. Standards containing from 0.1 to 0.5 µg P were prepared in a 2.5 ml volume. A distilled water blank was also prepared.

b) 0.4 ml of the combined reagent was added to each tube and the contents were mixed on a vortex mixer.

c) The color was allowed to develop at least 10 minutes but less than 30 minutes before the optical density was read against the blank at 880 nm.

d) The phosphate concentration was read off a standard curve.

Analysis of plasma samples.

a) 50 µl of distilled water was pipeted into each of several micorfuge tubes.

b) 10 µl of plasma, standard (0.5 µg P) or distilled water (blank) was added volumetrically.

c) 75 µl of 10% trichloroacetic acid was then added to each.

d) The precipitated protein was pulled down by centrifugation and 100 μl of the supernatent was transferred to test tubes containing 2. 5 ml of distilled water. The analysis then proceded as above for urine samples.

Note: The solution must be acidic when the combined reagent is added. This was checked with one drop of phenolphthalein. Each sample was acidic.

4. Analysis of ammonia concentration in urine (Nessler's reaction).

Reagent:

The Nessler's reagent was purchased from Scientific Products and contained 155 g/985 ml of solution.

Procedure.

a) 25 μl of urine was pipeted into 1 ml of distilled water.

b) 1 ml of Nessler's reagent was added and the color was allowed to develop for 10 minutes.

c) The samples and standards (containing 1, 2, 3, and 4 μg N as $(NH_4)_2SO_4$) were then measured for optical density against a distilled water blank on a colorimeter at 420 nm.

d) The concentrations were then read off a standard curve.

5. Analysis of glucose in urine and plasma.

Reagent:

Glucostat, Worthington Biochemical Corporation.

Standards.

a) Stock solution.

1.00% D-glucose. This was allowed to stand for 8 hours before use to permit mutarorotation to reach equilibrium. This precaution was necessary because the reaction is based on the enzymatic conversion of glucose to gluconic acid. The enzyme (glucose oxidase) is specific for β-D-glucose.

b) Working solutions.

Plasma standards: stock solution diluted to 300, 400 and 500 mg %.

Urine standards: stock solution diluted to 100 and 200 mg %.

Analysis of urine samples.

a) 10 μl of urine was added to each of several test tubes containing 2 ml distilled water.

b) 2 ml glucostat reagent was added to each at timed intervals.

c) After 10 minutes one drop of 4N HCl was added to each tube at the same intervals as in (b). This

stopped the reaction and fixed the color.

d) Standards and a reagent blank were prepared in the same way as the urine samples.

e) The optical density of each solution was read against the blank at 425 nm.

f) The concentration of glucose was calculated in each sample by comparison to the standard which most closely matched it.

Analysis of plasma samples.

a) 2.0 ml of distilled water was pipeted into each of several test tubes.

b) 25 µl of plasma was added to each of the tubes. Standards and a distilled water blank were prepared in the same way.

c) 1.0 ml of 2.0% $ZnSO_4 \cdot 7H_2O$ and 1.0 ml of 1.8% $Ba(OH)_2 \cdot 8H_2O$ were then added to each.

d) The precipitated protein was pulled down by centrifugation and 2.0 ml of the supernatant of each tube was transferred to another test tube. The analysis then proceded as above for urine samples.