THE EFFECTS OF DEHYDRATION ON *IN VITRO* TRANSPORT OF PHENOL RED IN RENAL TUBULES OF *PHRYNOSOMA SOLARE*

by

John James Corneveaux, III

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STATEMENT BY AUTHOR

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SIGNED: John J. Bumpers, III

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Wm. J. McCauley
W. H. McCAULEY
Professor of Zoology

August 19, 1964
Date

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W. F. McCauley--Professor of Zoology; University of Arizona

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ABSTRACT

This study involves an investigation of renal dye uptake as affected by dehydration. Kidney tubules of Phrynosoma solare were observed in a saline solution containing phenol red after various periods of dehydration. The time required to reach maximal dye concentration decreased as dehydration increased. After 20 days, the mechanism for dye transport apparently failed, and concentration time increased drastically. It is assumed that ADH affects these changes by altering the dilution factor of the dye.

INTRODUCTION

Certain species of horned-lizards (genus Phrynosoma) are known to avoid dehydration stress by confining periods of greatest activity to the cooler morning hours, and by burrowing into soft sand as the temperature reaches its maximum for the day (Heath, 1965). In addition to this behavioral mechanism for avoiding dehydration, some of these animals apparently possess physiological adaptations allowing them to undergo considerable dehydration without apparent deleterious effects. Specimens are reported to have lost 50% of their body weight through dehydration under experimental laboratory conditions, and to have remained in apparent good health (Roberts and Schmidt-Nielson, 1966).

The purpose of this study was to investigate the effects of dehydration on renal dye transport in Phrynosoma solare. Earlier studies have demonstrated that certain acidic and basic dyes are actively transported across renal tubule cell membranes in vitro and become concentrated against a gradient in the lumen (Forster, 1948; Forster and Taggart, 1950; Forster, Sperber and Taggart, 1954; Forster and Copenhaver, 1956; Hong and Forster, 1959). If dehydration would produce an observable change in concentration rate this would not only suggest a physiological mechanism for dehydration tolerance, but also would provide a valuable tool for subsequent studies of water balance in desert vertebrates. The development of methods for use of such dyes in water balance studies was a secondary purpose of this investigation.

METHODS

The method of investigation involved direct in vitro observation of renal dye uptake (Forster, 1948; Forster and Taggart, 1950; Hong and Forster, 1958). Phrynosoma solare was selected because of its high resistance to dehydration and ready availability.

Thirty specimens were collected and divided into equal groups. Group A was placed in an aquarium with about two inches of sand on the bottom and given a continuous supply of food and water; group B was placed in a similar aquarium, but given no food or water. After an initial confinement of four days specimens from both groups were removed, sacrificed and tested at two day intervals for a period of twenty-two days. Each time a group B specimen was tested, a group A specimen was given a similar test to provide a normal controlled comparison.

The following isotonic salt solution was prepared: (in millimoles per liter) NaCl 135, KCl 2.5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, NaH_2PO_4 0.5, and $NaHCO_3$ 10. Sodium bicarbonate was added as a dry salt with stirring after water was added to the mixture, and then 3×10^{-5} M of phenol red dye stirred in (Forster, 1948). For the sake of brevity, this solution will hereafter be referred to as Forster saline. The specimen to be studied was decapitated and the kidneys removed and teased. Following the scheme of Forster and Hong (1959) the freshly teased kidneys were placed in 5 ml of Forster saline contained in 1.2 X 4 cm plastic petri dishes into which oxygen was bubbled via 24 gauge hypodermic needles. At intervals of ten minutes, oxygenation was

interrupted while the dish was placed on a microscope stage to allow examination of the tubules (75X magnification). As will be described below, the tubules were observed periodically until maximal concentration of dye had been reached. All observations were carried out at room temperature.

Forster (1948) has demonstrated that renal tubules will actively concentrate certain dyes in vitro until these dyes are visually detectable in the lumina. Forster and Hong (1959) found that while several attempts have been made to determine quantitatively the amount of dye uptake, a highly reliable semiquantitative method is best. By observing the majority of tubules, an arbitrary assignment of concentration rating of "+" (definitely detectable) to "++++" (maximal) can be made. With practice, these arbitrary divisions prove to be clear cut and reproduceable.

Since antidiuretic hormone (ADH) is known to play a significant role in water retention during dehydration, an attempt was made to design a practical bioassay method to evaluate this phenomenon. After decapitation, each kidney from a group A specimen was placed in a separate petri dish--one dish to be used as the control and the other as a bioassay of the ADH in the pituitary from a specimen in group B. This was done by removing the pituitary for a group B specimen, crushing it in the 5 ml of Forster saline, and placing the second group A kidney into this solution. Observations were carried out as previously described.

In addition, to provide greater weight to the experimental findings, the kidneys from the group B specimen were observed in separate dishes. For a given test run, then, four separate dishes were

used--dish 1 contained a group A (control) kidney, dish 2 contained the second control kidney plus the crushed pituitary from the group B specimen, dish 3 contained a group B kidney, and dish 4 contained a second group B kidney as a check on the dish 3 observations. Thus, a comparison between the kidney tubule activity of normal and dehydrated specimens could be obtained plus the effects, if any, of the pituitary of dehydrated specimens on the normal kidney tubules.

Figure 2 illustrates the appearance of a typical distal tubule and collecting ducts following intake of dye to maximum concentration. It was by evaluation of such structures that concentration ratings were made. The distal tubules and collecting ducts were found to be concentrated in clusters at the superior end of the kidney as it lay in situ. By removing the capsule and teasing, the tubules were sufficiently separated to allow ample perfusion of the Forster saline and clear observation of the dye uptake.

RESULTS

ADH is known to play a large role in the passage of water across the membranes of the ascending loop of Henle and distal tubules. If little or no ADH is present in the kidney tissue, the tendency will be to produce an isotonic urine due to the free osmotic passage of water in the lumen according to a given osmotic gradient. Under these conditions, the filtrate passing from the so-called "salty tip" of the loop of Henle will absorb water from the surrounding interstitial area as the osmotic gradient lowers distally. (It is to be noted that this is an over-simplification of the urine forming process but is useful in defining the parameters of this study.) However, under various degrees of dehydration, ADH should be present in the kidney, and it should effectively make the membranes in question less permeable to water. Under conditions of lowered water permeability, free osmotic exchange will be restricted as the filtrate proceeds distally thus producing a more hypertonic urine.

Since phenol red is actively concentrated in the distal tubules, it seems reasonable to assume that the rate at which phenol red will concentrate in a tubule should be dependent upon the net water exchange occurring at a given time. Thus, in the absence of ADH, the movement of water into the tubule could tend to dilute a concurrent influx of dye particules resulting in a greater time span necessary to reach maximal concentration when compared with the tubule under the influence of ADH. If ADH is present in the pituitary of the dehydrated specimen, then placing this pituitary in the medium containing the normal control

tubules should cause a reaction similar to that observed in the dehydrated specimen--that is, a shorter period of time to reach maximal concentration. As dehydration progresses, the filtrate should become more and more concentrated due to water reabsorption efforts, and a more rapid rate of dye concentration would be observed. Based upon this preliminary supposition, the experiments were conducted as previously indicated.

At this point a definition of terms is necessary. For the purposes of this study, "concentration time" and "transport rate" are given separate meanings. "Concentration time" means the time required to reach a given observed color intensity of dye in the lumen. "Transport rate" refers to actual number of dye particles being moved across a given membrane. Obviously, if transport rate remains constant, dilute luminal conditions will result in a slower concentration rate than would be true for concentrated conditions.

The tubules of non-dehydrated control specimens required 60-80 minutes to reach maximal dye concentration, with 70 minutes being the average time required. After the first ten minutes, relatively few had reached the barely detectable "+" state of concentration. Even after more prolonged exposure (up to three hours), several specimens never went beyond the "+++" state.

As indicated by Figure 1, specimens dehydrated for progressively longer periods of time demonstrated a steadily decreasing concentration time until 18 days of dehydration at which time only 40 minutes were required to reach maximal concentration. However, this time remained the same at 20 days, and then increased rapidly until at 26 days the time had

increased beyond that required for the controls. Two specimens were tested at the 26 day stage, and both produced similar results.

The results of the pituitary study were not actually conclusive. In a number of instances, there was no observable difference between the normal kidney in standard Forster saline and the normal kidney in the Forster saline which contained the crushed pituitary. However, it was noted that there was a tendency for the control kidney to show a response shifted towards that of a dehydrated kidney when placed in Forster saline containing the pituitary. In about 50% of the pituitary tests, the otherwise normal tubules reached maximal dye concentration slightly faster than the control, usually in the range of 10-20 minutes faster.

DISCUSSION AND CONCLUSIONS

Robson (1963) draws a distinction between concentrating and diluting kidney--that is between kidneys which are capable of producing hypertonic urine and those which produce hypotonic urine. In the case of Phrynosoma, Roberts and Schmidt-Nielson (1966) found that there was a total inability to produce hypotonic urine from the kidneys, even under water loading conditions. In fact, Roberts and Schmidt-Nielson demonstrated through electron microscope studies that Phrynosoma distal tubule cells are generally lacking the characteristic elongated mitochondria found in many animals. This, they feel accounts for a low rate of sodium transfer, and the inability to produce hypotonic urine. Therefore, in the Robson sense (op. cit.), Phrynosoma kidneys could be classed as concentrating kidneys.

Since sodium transfer is an energy-dependent process, it follows that such a process would be inhibited in a region where energy producing mitochondria were absent as in Phrynosoma distal tubules. However, it has been demonstrated that these same tubules will accumulate a high concentration of phenol red against a definite gradient, which immediately suggests an energy-dependent process. That dye concentration is an energy-dependent process was demonstrated by Forster and Hong (1958) when tubules which had accumulated a maximal dye concentration were subjected to such metabolic inhibitors as cold and 2,4 dinitrophenol. Under such conditions the accumulated dye was observed to run out of the tubules, indicating that once the energy sources needed for active transport were

inhibited, dye diffused along its own concentration gradient. Therefore, it is difficult to explain active transport of phenol red in Phrynosoma distal tubules except by suggesting that another unknown energy producing mechanism is present.

It is tempting to explain this problem by assuming that perhaps the dye transport actually takes place in the proximal tubules and is retained in the distal lumina due to membrane impermeability to the dye in that region, thus allowing it to become more concentrated. However, while it may be possible that some dye transport does indeed occur in the proximal tubules, several distal tubules were completely separated during the course of this investigation, and even as isolated tubules, dye was concentrated as before. No dye concentration was noted in the proximal tubules. Therefore, it must be assumed that the major site of dye transport and concentration is in the distal tubules.

The single most important finding of this study is that the time required for maximal dye concentration decreases as dehydration progresses. However, it is not known whether the decreasing dye concentration time during dehydration reflects an increasing transport rate across the membrane or a simple dilution factor as discussed earlier. It seems more likely that the transport rate remains relatively constant and that the increasing impermeability of the membrane resulting from an increasing ADH titer allows for a relatively shorter dye concentration time. The fact that the presence of the dehydrated pituitary in the solution containing the normal kidney tended to cause a slight shift to the dehydrated characteristics gives some support to this theory.

Since after 20 days of dehydration the specimens were moribund (one had lost considerable motor control and tended to become convulsive when touched), it is reasonable to assume that metabolism was failing at this stage. The concentration rate curve (Fig. 1) indicates a rapid drop during this same period which suggests a possible failure in the dye transport mechanism. This might be explained in one of two ways (possibly both): (1) ADH production falls off as metabolism begins to fail; (2) energy producing mechanisms necessary for active transport in the tubule begin to fail as dehydration and starvation become more acute.

Because it is not possible to draw any definite conclusions concerning the mechanistic aspects of this study, it is obvious that there are several points which are in need of further investigation. Most obvious is the need to explain the source of energy for active dye transport in view of the general lack of energy producing mitochondria. At the same time one would need to determine whether the actual transport rate of dye particles changes or remains static as dehydration progresses. Another point which needs further study is whether ADH does indeed play an active role in the rate changes noticed during dehydration. While there are tempting indications that this might be so, certainly no conclusions can yet be drawn. Finally, since ADH is a general term given to a number of hormones known to affect membrane permeability to water, it would be useful to isolate and identify the actual chemical involved as the antidiuretic hormone in Phrynosoma solare.

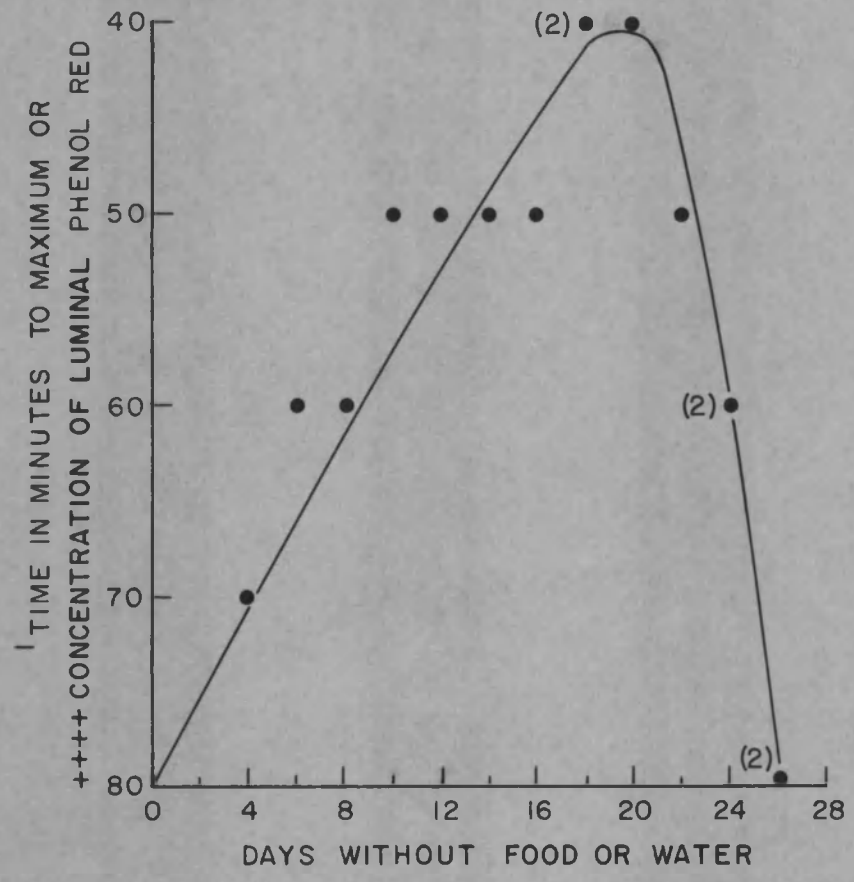
The two major objectives of this study have been satisfied, however. It has been clearly demonstrated that there is a definite

change in renal concentration rate of phenol red during dehydration. Since this is a clearly visible phenomenon *in vitro* the use of renal dye transport as an investigational tool in water balance studies has been demonstrated to be of definite potential.

SUMMARY

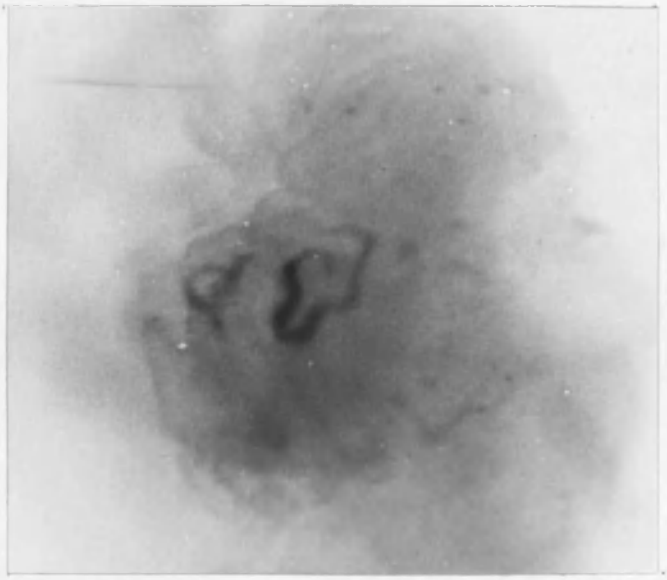
1. The purpose of this study was to investigate the effects of dehydration of renal dye uptake in Phrynosoma solare and to determine the usefulness of renal dye uptake as an investigative tool in such studies.
2. In vitro observations of phenol red uptake in distal tubules were made during various stages of dehydration.
3. As dehydration became more pronounced, the time required for maximal dye concentration decreased until 20 days of dehydration, at which point a rapid reversal in concentration rate occurred.
4. The changes noticed in dye concentration rate are believed to be the result of changes in dilution factors due to changes in ADH titer as dehydration progresses.

APPENDIX



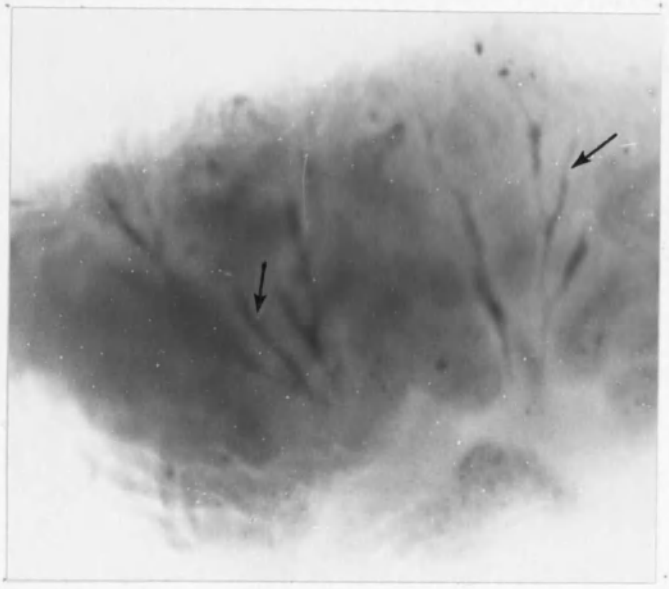
[1]Readings were taken only at ten minute intervals -- each position on chart indicates one pair of kidneys. Where (2) appears, two specimens of equal dehydration time were tested. Line is drawn in for illustrative purposes.

Figure I. Maximal dye concentration rates as affected by dehydration period.



Photograph of typical distal tubule at maximum concentration (++++).

PLATE I



Photograph of collecting ducts at maximum concentration (++++). Arrows indicate general areas of evaluation as discussed in text.

Note: Fuzzy appearance of photographs is due to overlying tissue.

PLATE II

Figure 2. Typical examples of luminal dye concentration.

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