AN ABSTRACT OF THE THESIS OF

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Title: MECHANISMS OF WATER MOVEMENT INTO AMPHIBIANS WITH SPECIAL REFERENCE TO THE EFFECT OF TEMPERATURE

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The rate of osmotic movement of water across the skin of Rana pipiens, in vivo and in vitro, was measured at 5° C and 15° C. With 23 mM/l sucrose bathing the epithelium the osmotic flow into intact animals was 1.40 $\mu l/cm^2$-hr at 5° C and 2.62 $\mu l/cm^2$-hr at 15° C (Q_{10} = 1.9). The osmotic flow across isolated skin (23 mM/l sucrose outside, Ringer's solution inside) was 1.3 and 2.0 $\mu l/cm^2$-hr at 5° C and 15° C respectively (Q_{10} = 1.5).

The osmotic movement of water, J_F^{OS}, in both in vivo and in vitro skin consists of two components, diffusive flow, J_F^D, and hydrodynamic flow J_F^H. Tritiated water (THO) was used to measure J_F^D and J_F^H was calculated, ($J_F^H = J_F^{OS} - J_F^D$), after correcting for the gradient. The units are $\mu l/cm^2$-cm $H_2O \times 10^5$. For in vivo skin J_F^D was 9.2 at 5° C and 15.4 at 15° C. For in vitro skins

J_F^D was 9.2 and 12.9 at 5° C and 15° C respectively. The values for J_F^H for *in vivo* skins were 20.6 and 38.5 and for *in vitro* skins 28.4 and 18.4 at 5° C and 15° C respectively. Temperature does not appear to have a direct effect on the permeability of *in vitro* skin since the observed Q_{10}'s can be accounted for by changes in the viscosity of water. However, the effect of temperature on both the diffusive and hydrodynamic flow in *in vivo* skin cannot be explained in these terms. The causes of these changes were investigated.

Evidence is presented that temperature does not exert its effect indirectly by changing the titer of the neurohypophysial hormones, which would affect the radii of the pores (or channels) in the skin. The temperature effect on *in vivo* skin was not abolished after injection of vasopressin. Analysis of J_F^{OS}/J_F^D in the absence and presence of vasopressin indicate that temperature does not exert its effect on pores. This conclusion is also supported by measurements of J_F^{OS}/J_F^D on the salamander *Ambystoma gracile*, where the neurohypophysial hormone does not affect the skin. A large Q_{10} for J_F^{OS} and J_F^D was found but the ratio did not change indicating that there was no change in the radii of the pores in the skin.

It is postulated that temperature affects the osmotic flow of water in *in vivo* skin by causing changes in circulation or internal mixing and thus affecting the concentration gradient. The physiological significance of this type of control over water uptake is discussed

in terms of the energetics of osmoregulation.

In addition to an osmotic component to water movement there is also a non-osmotic component in the presence of salts in the external bath. The non-osmotic component was 30 percent of the total flow at 5^{o} C and 25 percent of the total flow at 15^{o} in *in vivo* skin (1/10 Ringer's outside). The non-osmotic component in *in vitro* skin (1/10 Ringer's outside, Ringer's inside) was 30 percent of the total water flow at 15^{o} C; a measurable non-osmotic component was not obtained at 5^{o}.

Mechanisms of Water Movement into Amphibians with Special Reference to the Effect of Temperature

by

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INTRODUCTION

Historical Review

The development of the microcope allowed Robert Hooke (1665) to describe cork as being composed of "pores of cells, (which) were not very deep, but consisted of a great many little Boxes, separated out of one continued long pore, by certain Diaphragms....." Even though this first description of cells was very suggestive it was not until the 19th century that Schultze and Kühne proposed that a membrane separates the protoplasm of the cell from the external solution (Schultze, 1863; Kühne, 1864, In: Troshin, 1966). These workers were interested in the nature of protoplasm and realized that it was an aqueous solution of organic (principally protein) andmineral substances which was immiscible with the surrounding solution. It was to explain this that each of them postulated some kind of a barrier separating the inside and the outside of the cell. Kühne suggested that the barrier was made of coagulated protein while Schultze thought it was a condensed layer of protoplasm itself.

The concept that this barrier or membrane is permeabile arose from the investigations of Pfeffer and de Vries (Pfeffer, 1877; de Vries, 1884, 1885, 1888, In: Troshin, 1966) who observed that plant cells behave as osmometers when immersed in certain

solutions. Pfeffer proposed that the membrane is highly permeable to water and only slightly, or not at all, permeable to the molecules of water soluble substances. Overton (1902), after studying the penetration of more than 500 substances into cells, developed the lipid theory of cell permeability. He proposed that the plasma membrane was a film of fat-like substance which prevented the protoplasm from mixing with the water of the surrounding medium.

Ruhland and Hoffman (1925) subsequently presented evidence for an ultrafilter theory of permeability. In their experiments they demonstrated that for a large number of substances there is a correlation between the fall in magnitude of the threshold plasmolytic concentration (which served as their measurement of permeability) and the increase in the magnitude of the molecular refraction (which indicates molecular size). At the same time, they observed no correlation between the magnitude of the distribution coefficient between ether and water and the threshold plasmolytic concentration, which seemingly contradicted Overton's theory.

Collander and Bärlund (1933), on the other hand, found an increase in permeability for a homologous series of substances with an increase in the number of carbon atoms, supporting Overton's theory. These authors also found a group of substances which did not increase their penetration rate with an increase in the distribution coefficient between olive oil and water. They therefore combined the

two theories into a lipid-filtration theory. This theory stated that the rate of penetration of substances insoluble in lipids is regulated by the size of the pores in the membrane, while the rate of penetration of substances soluble in lipids is governed by the distribution coefficient between the non-aqueous and aqueous phases.

These basic ideas have not changed although much work has been done in trying to deduce the actual structure of the membrane. Harvey (1931) and Cole (1932) measured the surface tension between the cell membrane and the aqueous surroundings and found it was about 0.1 dyne/cm. Since no lipids are known which could have a low solubility with water and at the same time a low surface tension, it became evident that some other substance besides lipid was present in the membrane. Danielli and Harvey (1935) found that the cause of the lower tension was the presence of protein. This and the data of Gorter and Grendel (1925) which indicated that there is sufficient lipid in the red blood cell membrane to make a layer two molecules thick, led Davson and Danielli (1943) to propose a protein-lipid bilayer model for the cell membrane.

There has been much criticism of this model. Biochemical studies of the behavior of cell membranes in the presence of various lipases and organic solvents have indicated that lipids are on the outer surface (Lenard and Singer, 1968), while Davson and Danielli's model has the lipid enclosed in protein. The only direct experimental evidence

for the model has come from electron micrographs which show a trilaminar image of membranes when they are fixed with osmium tetroxide. However, the interpretation of the micrographs has been criticised and it seems as though a reevaluation of these micrographs is required (Korn, 1969).

At present all we know is that the membrane is composed of protein and lipids, the rate of penetration of lipid soluble substances through the membrane is determined by their lipid-water partition coefficient, and the rate of penetration of lipid insoluble substances is determined by their molecular size. This last phenomena is interpreted as meaning that pores are present in the membrane.

The water permeability of the external epithelia of fresh-water animals was first extensively studied by Paul Bert (1871a; 1871b). He noticed that frogs lose weight when placed in sea water and proposed that their loss is due to a "desiccation" of the tissues due to the loss of water. Since that time there has been an extensive study of osmo-regulation in aquatic animals. It was not until the 1930's, however, that Homer Smith (1932) in New York and August Krogh (1939) in Copenhagen set down some of the basic principles of osmo-regulation and stimulated work which has continued to the present.

A Review of Current Theories of Water Movement through Membranes

Osmotic Movement through Pores

Studies of water permeability of both cell membranes and epithelial tissue indicate the presence of pores or channels through which lipid-insoluble substances may pass. Flow through an epithelial membrane may either be through the cells comprising the membrane or between the cells. The interpretation of water flow through epithelial membranes has been used successively to explain water flow through cell membranes (Solomon, 1968). Likewise, concepts of flow through cell membranes have been applied to epithelia (Diamond and Tromely, 1966; House, 1964). Thus, the following analysis of water flow will apply to both cell membranes and epithelial tissue.

The rate of osmotic water flow across the external epithelia of an animal is usually expressed mathematically as:

$$J_V^{OS} = P_{OS} A_m (\Delta \pi) \qquad (1)$$

where:

J_V^{OS} = the osmotic flow of water ($ml \cdot sec^{-1}$)

A_m = the total membrane area (cm^2)

$\Delta\pi$ = the difference in osmotic pressure (cm-H_2O)

P_{OS} = the permeability of the membrane (cm-sec^{-1}-cm H_2O^{-1}).

Flow through a porous membrane can also be expressed as follows (Pappenheimer, 1953):

$$J_V^{OS} = k \frac{A_p}{\Delta X} (\Delta\pi) \qquad (2)$$

where:

A_p = cross-sectional pore area (cm^2)

ΔX = path length through the membrane (cm)

k = constant of proportionality

Included in k are the frictional restrictions imposed by the membrane and thus k will be specific for each membrane. Equating equations 1 and 2 and solving for P_{OS} gives:

$$P_{OS} = \frac{A_p}{A_m} \frac{1}{\Delta X} k \qquad (3)$$

The term $\frac{A_p}{A_m}$ is the ratio of the total pore area to the total membrane area and represents the geometrical restrictions introduced by the fact that the area available for water flow is limited to a portion of the total membrane area. The fact that the driving force is the concentration gradient (actually the chemical potential gradient which is closely approximated by the concentration gradient in dilute solutions), and not just the difference in concentration, is represented by the term $\frac{1}{\Delta X}$. However, since the term ΔX can not be easily

measured in biological membranes and is usually considered to be constant it is incorporated into the permeability constant P_{OS}.

The factors entering into k will depend upon the mechanism of flow. Osmotic flow may result from diffusional flow and/or bulk flow of solvent through pores (also called laminar flow or hydrodynamic flow). If the flow is by diffusion through pores Chinard (1952) has shown that it can be expressed as follows:

$$J_V^D = \frac{D_{H_2O}\,\overline{V}_{H_2O}}{RT}\,\frac{A_p}{\Delta X}\,(\Delta \pi) \qquad (4)$$

where

J_V^D = the diffusion flow ($ml\text{-}sec^{-1}$)

R = the gas constant ($ergs\text{-}mole^{-1}\text{-}deg^{-1}$)

D_{H_2O} = free diffusion coefficient of water in water ($cm^2\text{-}sec^{-1}$)

T = absolute temperature (deg)

$\overline{V}$ = partial molar volume of water ($ml\text{-}mole^{-1}$)

Thus for diffusional flow $k = \frac{D_{H_2O}\,\overline{V}_{H_2O}}{RT}$. If the flow is hydrodynamic through pores the expression would be:

$$J_V^H = \frac{r^2}{8\eta}\,\frac{A_p}{\Delta X}\,(\Delta \pi) \qquad (5)$$

where:

J_V^H = is the hydrodynamic flow ($ml\text{-}sec^{-1}$)

η = viscosity of the fluid (dynes-sec-cm^{-2})

r = the radius of the pore (cm).

Here $k = \frac{r^2}{8\eta}$.

When J_V^D is measured with isotopic water (THO) under conditions of no net flow, $\Delta\pi = \Delta C_{H_2O} RT$, then equation 4 can be changed as follows:

$$J_V^D = D_{H_2O} \overline{V}_{H_2O} \Delta C_{H_2O} \frac{A_p}{\Delta X} \qquad (6)$$

With THO on one side and no THO on the other the concentration gradient will be 55.5 M then $(\overline{V}_{H_2O} \Delta C_{H_2O}) \tilde{=} 1$ and equation 6 reduces to:

$$J_V^D = D_{H_2O} \frac{A_p}{\Delta X} \qquad (7)$$

Mauro (1957), Koefoed-Johnsen and Ussing (1953) and Pappenheimer (1953) have measured J_V^D with isotopic water across artificial and biological membranes and thus obtained a value for $\frac{A_p}{\Delta X}$ which can be substituted into equation 4. If the rate of movement from an osmotic gradient is from diffusion then, knowing $\frac{A_p}{\Delta X}$, the theoretical rate of flow J_V^D can be calculated for any osmotic pressure difference using equation 4. However, the theoretical values are always lower than the actual values of J_V^{OS} measured

when there is an osmotic pressure difference. The theoretical value was three to five times lower in the experiments of Hevesy, Hofer and Krogh (1935) on frog skin and several hundred in the work of Robbins and Mauro (1960) on artificial membranes.

Pappenheimer (1953) and Koefoed-Johnsen and Ussing (1953) attributed this difference to the fact that there is a laminar flow, as well as a diffusional flow during osmotic movement of water.

If one assumes that the two flows are additive then the osmotic flow (J_V^{OS}) will be given by:

$$J_V^{OS} = J_V^H + J_V^D \tag{8}$$

One can then calculate the fraction of the flow that will result from diffusion at any pore size from equations 4 and 5 as shown by Pappenheimer's (1953) equation:

$$\frac{J_V^D}{J_V^H + J_V^D} = \frac{1}{1 + \frac{r^2 RT}{8\eta D_{H_2O} \overline{V}_{H_2O}}} \tag{9}$$

This equation shows that as the pore radius gets larger the diffusive fraction gets smaller. When the pore size reaches about 20 Å the flow will be almost entirely hydrodynamic. This is in the range of capillary pore sizes which are about 30-40 Å (Pappenheimer, 1953). Membranes of cells and less porous tissue such as skin have pores

which are smaller so that across these membranes diffusion plays an increasingly important role. The work of Solomon and co-workers (1968) support the above hypothesis that the reason for the discrepancy between the isotopic diffusion and the total flow under osmotic conditions is that there are really two flows occurring in the small pores, diffusive and laminar or hydrodynamic. He has used several methods to estimate the pore size in red blood cells and all give a value close to the 4.5 Å value predicted from considerations of hydrodynamic and diffusive flow.

Dainty (1963) has given an explanation for the evidence that osmotic flow is partially or wholly a hydrodynamic flow as would be created by a hydrostatic pressure. He assumes that the mechanism of semi-permeability is the exclusion of the solute molecules from the pores in the membrane, thus the pores are filled with water only. From the consideration of irreversible thermodynamics, which states that the fluxes are linear functions of all the forces, the basic equation of water flow in the differential form is:

$$\phi_w = L_{ww} \left[-\overline{V}_w \frac{dP}{dX} - RT \frac{d}{dX} (\ln N_w) \right] \qquad (10)$$

where:

ϕ_w = the water flux (moles-cm^{-2}-sec^{-1})

L_{ww} = the Onsager coefficient relating the fluxes to the forces (mole^2-Joules^{-1}-cm^{-3}-sec^{-1})

$\overline{V}_w$ = the molar volume ($cm^3 mole^{-1}$)

$\frac{dP}{dX}$ = the pressure gradient ($Joules\text{-}cm^{-4}$)

R = gas constant ($Joules\text{-}mole^{-1}\text{-}deg^{-1}$)

T = absolute temperature (deg)

N_w = mole fraction of water

Under equilibrium conditions, $\phi_w = 0$, then:

$$\overline{V}_w P + RT \ln N_w = \text{constant} \qquad (11)$$

This means that at the pore entrance where there is an increase in N_w there must be a decrease in the pressure. This is shown in Figure 1.

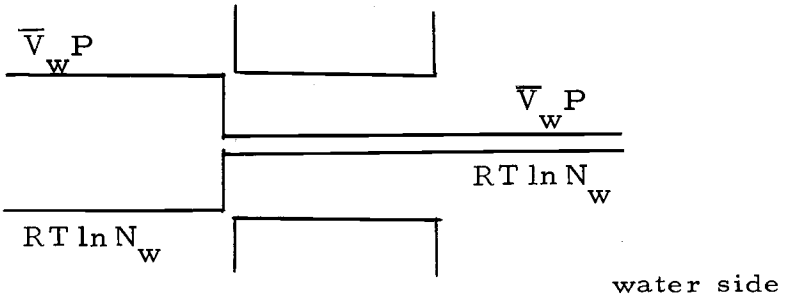


Figure 1. Change in the mole fraction and pressure through a water filled pore at osmotic equilibrium.

What happens when there is flow through the pore? Flow from pure hydrostatic pressure is easily depicted and is shown in Figure 2.

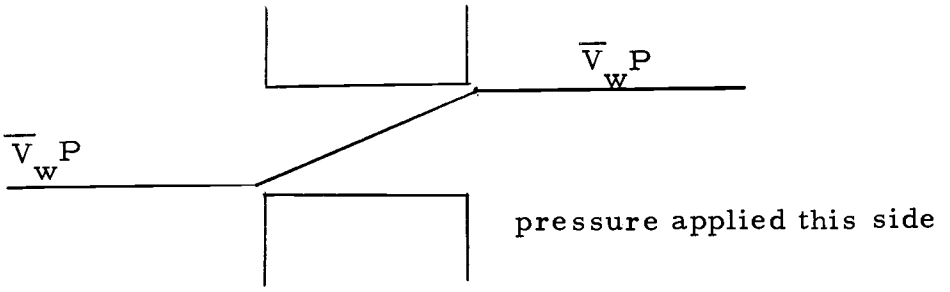


Figure 2. Pressure gradient through a water filled pore due to a difference of hydrostatic pressure.

When there is no hydrostatic pressure but only a mole fraction difference Dainty shows the mole fraction and pressure profiles as seen in Figure 3.

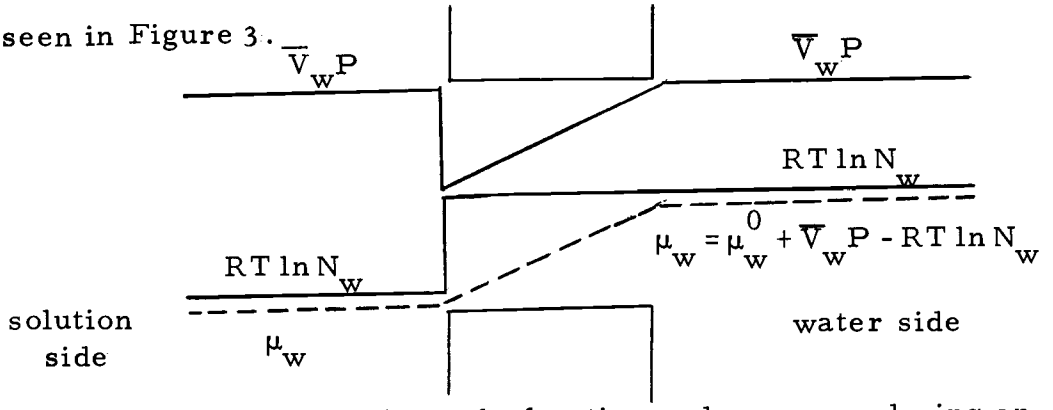


Figure 3. Changes in the mole fraction and pressure during an osmotic flow.

The mole fraction is as shown because it is assumed that the pores are filled with pure water. There is a decrease in the pressure on the solution side resulting in a pressure change the same as when a difference in hydrostatic pressure exists. The drop in pressure on the solution side results from the lower density of molecules, because the solute molecules cannot penetrate the pore.

Non-osmotic Flow

The osmotic flow through small (< 20 Å) pores is then best explained as a combination of hydrostatic flow and diffusive flow. However, in addition to an osmotic movement of water there is also a so-called non-osmotic component, i.e., a net flux of water in the absence of an osmotic or hydrostatic gradient (Ried, 1892; Kirschner et al., 1960; Diamond, 1962; House, 1964). Thus:

$$J_V^T = J_V^D + J_V^H + J_V^{NOS} \qquad (12)$$

where J_V^{NOS} is the non-osmotic flow.

The non-osmotic movement of water has been attributed to active transport of water (Fischer, 1955; Ullman et al., 1960). However, the more common interpretation is that the apparent non-osmotic movement of water in fact involves active transport of solute and the creation of osmotic imbalance either in the membrane or across it (Curran, 1960; Diamond, 1962; House, 1964). It is noteworthy that the non-osmotic movement of water across frog skin is abolished if ions are removed from the solution bathing the epithelium (Kirschner et al., 1960). Numerous models have been proposed to explain movement of water in the absence of, or even, against, an osmotic gradient. That of Curran (1960), has been applied to a number of epithelia. The model is based on the idea that water movement

will be differential across membranes if their pores differ in radius. Consider an epithelium consisting of two membranes in series, one with small pores and one with larger pores as shown in Figure 4.

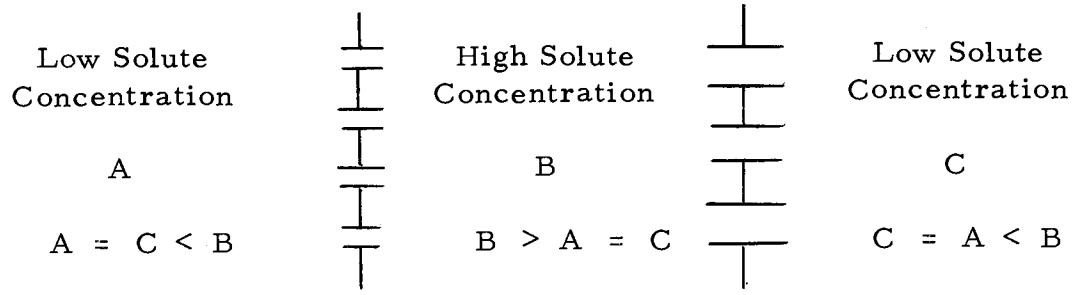


Figure 4. Curran's model for non-osmotic water flow. Flow will be from A to C.

Active solute transport will create and maintain a higher solute concentration in B than in A. Water will move from A to B and cause an increase in the hydrostatic pressure in B. There will be no movement of water from C to B because of the large pores. Staverman (1952) has shown that for membranes which are permeable to solute the actual osmotic pressure will be less than the theoretical by a factor which depends upon the size of the pores. Durbin (1960) has shown that for relatively small solutes and large pores the osmotic pressure is almost zero. Curran and Macintosh (1962) have tested Curran's (1960) hypothesis by using a cellophane membrane (with small pores) and sintered glass (with large pores). Under these conditions a significant water movement was found in the absence of a concentration gradient.

More recently, House (1964) has proposed a slightly different theory for the non-osmotic flow across frog skin. Koefoed-Johnsen and Ussing (1958) proposed that the ion pumps in frog skin are located on the inner wall of a single layer of cells. The outer membrane of these cells is assumed to be permeable to Na^+ but not K^+ and the inner membrane the reverse (MacRobbie and Ussing, 1961). The pumps extrude Na^+ and accumulate K^+ keeping the inside of these cells low in Na^+ and high in K^+. The actual osmotic pressure across a membrane permeable to solute and solvent is related to the theoretical osmotic pressure by the reflection coefficient, σ (Staverman, 1952):

$$\sigma = \frac{\text{actual osmotic pressure}}{\text{theoretical osmotic pressure}} \tag{13}$$

With this information House expressed the flow across the outer and inner membrane as follows:

$$J_V^O = L_p^O RT [C_K + C_i - \sigma_{Na}^O C_{Na}] \tag{14}$$

$$J_V^i = L_p^i RT [C_{Na} - C_i - \sigma_K^i C_K] \tag{15}$$

where:

J_V^O = net volume flow across outer membrane ($cm^3 \cdot cm^{-2} sec^{-1}$)

J_V^i = net volume flow across inner membrane ($cm^3 \cdot cm^{-2} \cdot sec^{-1}$)

L_p^O = hydraulic conductivity of outer membrane ($cm \cdot sec^{-1} atm^{-1}$)

L_p^i = hydraulic conductivity of inner membrane ($cm \cdot sec^{-1} atm^{-1}$)

C_K = osmolarity of KCl in central compartment (moles cm^{-3})

C_i = osmolarity of impermanent potassium salt in central compartment (moles cm^{-3})

C_{Na} = osmolarity of NaCl in external Ringer solutions (moles cm^{-3})

σ_{Na}^O = reflection coefficient of outer membrane for NaCl

σ_{Na}^K = reflection coefficient of inner membrane for KCl.

Even when the concentrations on the inside and outside of the membrane are equal there will be a net flow across the outer and inner membrane. This is because, even though in equation 14, $C_K + C_i = C_{Na}$ the actual osmotic concentration is $\sigma_{Na}^O C_{Na}$ which is less than $C_K + C_i$ thus there is a net flow of water into the cell. The same reasoning holds for equation 15 expressing the flow across the inner membrane.

Effect of Temperature on Water Movement

Examination of equation 5 shows that temperature would be expected to change the hydrodynamic flow because of its effect on viscosity and its effect on $\Delta\pi$ ($\Delta\pi = RT\Delta C_S$). The viscosity is decreased by 30 percent with an increase in temperature from 5° C to 15° C (Wang et al., 1953). The increase in the absolute temperature would be $\frac{10}{278} \times 100\% = 3.6\%$. Since J_V^H is indirectly related to viscosity and directly related to absolute temperature the increase in J_V^H with an increase in temperature from 5° C to 15° C would be about 35 percent. The effect of temperature on the diffusive flow is the same as on the hydrodynamic flow since the diffusion coefficient is also increased by 30 percent due to the change in viscosity as shown by the Stokes-Einstein equation:

$$D_{H_2O} = \frac{RT}{6\eta_{H_2O} a_{H_2O} \pi^*} \tag{16}$$

where

a_{H_2O} = radius of the diffusing molecule in $\mathring{A}$

π^* = 3.14

Equation 4 then shows that the diffusion flow J_V^D would be expected to increase by 35 percent. Since J_V^H and J_V^D are assumed to be additive, the total osmotic flow J_V^{OS} would be expected to increase by 35 percent with a temperature increase from 5° C to 15° C.

The date for the effect of temperature on the total water flow (J_V^T) across capillaries (Pappenheimer, 1953), invertebrate nerve

fibers (Nevis, 1958) and human red blood cells (Jacobs et al., 1936; Hempling, 1960) have been explained as resulting from viscosity changes in water.

However, Hempling (1960) working on Ehrlich ascite tumor cells and Lucké and McCutcheon (1932) working on Arbacia eggs obtained results for the temperature effect on the total net flow of water which are too large to be explained by changes in viscosity. Likewise, Hays and Leaf (1962) measured the THO diffusion rate through toad bladder and found much larger changes due to temperature than could be accounted for by changes in viscosity. They also found that with the addition of ADH the effect of temperature decreased to what would be predicted from changes in viscosity. They attributed their results to one of two possibilities: (a) that there are very small pores so that there is more interaction between the membrane and water than between water and water; the addition of ADH would then increase the pores and reduce the membrane water interaction, and (b) that there is a large layer of highly structured or bound water through which water passes and that the addition of ADH decreased this layer reducing the interaction of the diffusing water with the bound.

A large effect of temperature on the rate of osmotic uptake of water (J_V^T) is also seen in whole animals. In most aquatic vertebrates there is an increase of 100 percent or more for a 10° C rise

in temperature which is much larger than the 35 percent increase in rate expected from the effects of temperature on viscosity. This has been shown in the lampray (Wikgren, 1953), white sucker (Mackay and Beatty, 1968), bullfrog (Schmidt-Nielsen and Forster, 1954) and a urodele (Parsons and Alvarado, 1968).

Objectives of Study

Most of the above studies have been done on artificial membranes or isolated biological membranes. While studies of this type are very informative the models often have to be modified when applied to whole animals. A good example is the extensive work that has been done on isolated frog skin by Ussing and co-workers in Copenhagen (Ussing and Zerahn, 1951; Koefoed-Johnsen and Ussing, 1958; Ussing, 1966). They have shown that the transport of sodium across isolated skins bathed on both sides with Ringer's solution is active, whereas transport of Cl, under these conditions is passive. Yet is has been shown many times that intact frogs can transport chloride actively even in the absence of Na^+ transport (Krogh, 1937; Jørgensen et al., 1954; Dietz et al., 1967).

Little is known about the mechanism of movement of water into whole animals. It was thus decided to determine the relative importance of the various types of water flow: diffusive, hydrodynamic and non-osmotic, in whole animals and compare this with isolated

skins.

A second objective was to study the effects of temperature on water movement across the skin of intact animals and isolated skins. This was done for two reasons. First, in order to obtain information about the process of water movement across frog skin and second, to gain insight into the physiological mechanism, whereby the whole animal regulates its water content by adjusting its uptake of water.

MATERIALS AND METHODS

Animals

Rana pipiens were obtained from a commercial source in Wisconsin and kept at 10° C in a container of dechlorinated tap water which was tilted so that half of the container remained dry. Two groups of frogs were used; one was an older, larger (70-100 g) group, obtained and used during the winter months (October, 1968-April, 1969), and a second, younger and smaller (40-60 g) group was obtained and used during the spring (May, 1969-July, 1969). The latter group will be referred to as spring frogs and the former as winter frogs. To insure uniform hydration, the animals were forced to remain in dechlorinated tap water for 12 hours prior to each experiment. Larval Ambystoma gracile were collected in the vicinity of Corvallis, Oregon. The animals were kept in polyethylene containers at 8° C. All animals were acclimated for at least five days at the temperature under consideration.

Whole Animal Measurements

The osmotic uptake of water into frogs and salamanders was measured by blocking the cloaca of animals with a purse string ligature and weighing them at one hour intervals, for four hours, on a Mettler balance (precision ± 0.05 g). Prior to ligation the bladder was emptied by suprapubic compression.

The rate of water uptake, determined from the rate of weight increase, was linear.

Diffusion measurements were made by placing individual frogs in a 600 ml beaker containing 10 μc of Tritiated water (New England Nuclear) in 200 ml of test solution. The beaker was shaken at a frequency of 2 cycles/sec in a constant temperature water bath. Samples were taken every ten minutes for one hour. One hundred microliters (μl) of the bath was added to 1 ml of water and 10 ml of Triton-X counting media (Patterson and Greene, 1965). Samples were counted to a total of 20, 000 counts on a Nuclear-Chicago ambient temperature liquid scintillation counter. The external standard method was used to estimate quenching.

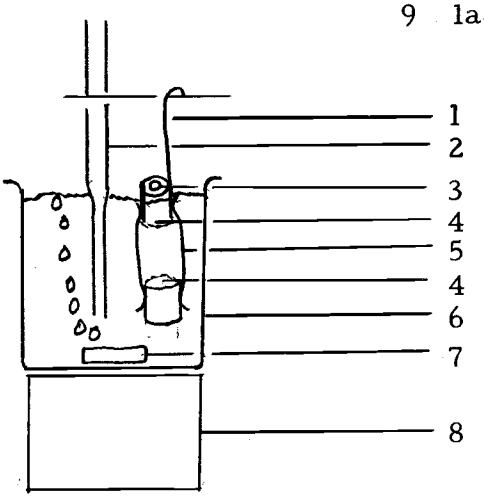
Injections of antidiuretic hormone (ADH) were made using Pitressin, a commercial product of Park-Davis Company. The hormone was obtained in ampoules with 10 pressor units in 0. 5 ml and was diluted with Ringer's solution so that the injection volume was 0. 25 ml/animal. The hormone solution also contained chlorobutanol and acetic acid. Intraperitoneal injections were made into unanesthetized animals. For osmotic uptake experiments animals were injected at zero time; for the diffusion experiments, the injection was made one hour prior to the beginning of the diffusive measurement.

In vitro Skin Measurements

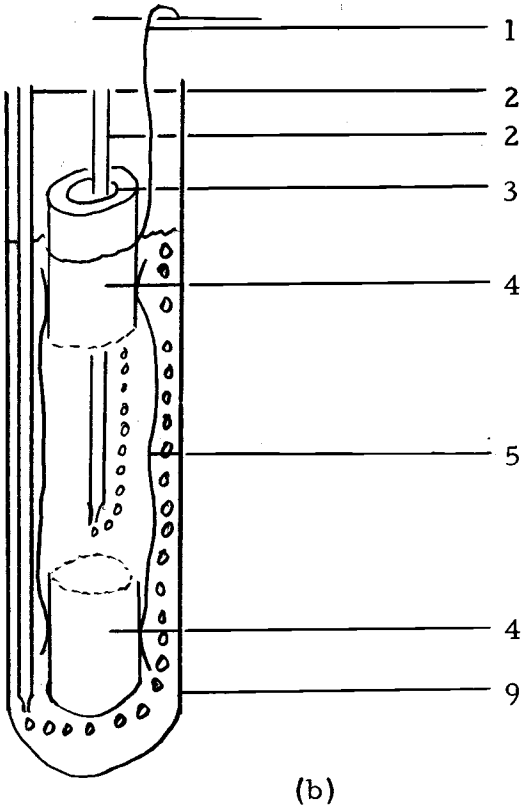
To measure the water movement across isolated skin, the skin from the calf region of frogs was removed. Acrylic plugs, one of which had a hole in the center for sampling, were inserted into each end making a bag with the outside (epithelial) of the skin facing inward. The osmotic movement of water was measured by filling the bag with 1/10 Ringer's solution (23 mOsmo/l) or sucrose (23 mOsmo/l). The bag was suspended in Ringer's solution (225 mOsmo/l, 111 mM/l sodium chloride, 2 mM/l potassium chloride, 1.8 mM/l calcium chloride and 2.4 mM/l sodium bicarbonate) at the desired temperature. The Ringer's solution was mixed with a magnetic mixing bar and well aerated (Figure 5a). The bags were equilibrated for one hour and then weighed at two hour intervals for eight hours. Precision of the method was ± 3.8 mg (20 successive weighings). The equilibrium was necessary because skins take up water during the first hour (Kirschner *et al.*, 1960).

For diffusion experiments, either sucrose (225 mM/l) or Ringer's solution was placed inside the bag and the bag placed in 20 ml of Ringer's solution in a large test tube. Mixing was done by aerators in the external and internal solutions (Figure 5b). To the inside of the bag 25 µl of THO (1.5 µc) was added. One minute later 100 µl was removed for determination of the radioactivity. Samples

1 hook
2 aerator
3 sampling hole in top acrylic plug
4 acrylic plug
5 frog skin
6 600 ml beaker
7 magnetic stirring bar
8 magnetic stirring motor
9 large test tube



(a)



(b)

Figure 5. Diagram of the apparatus used to measure (a) osmotic movement of water and (b) THO diffusion through isolated skin.

were then removed from the outside solution every 20 minutes and counted.

The area of each skin bag was determined at the end of each experiment by tracing the outline on graph paper. The osmotic pressure of each solution was checked on a Mechrolab vapor pressure osmometer.

Treatment of Data

THO Flux Determination

The equation used to determine the rate of diffusion of water was derived by Paganelli and Solomon (1957). The equation used, when the decrease in radioactivity (THO added to compartment p and compartment p sampled) was measured, was:

$$\ln (p - p_\infty) = -kst + \ln (p_o - p_\infty) \qquad s = \frac{p_o}{v_q p_\infty} \tag{17}$$

when the increase in activity (THO added to compartment q and compartment p sampled) was measured the equation used was:

$$\ln (p_\infty - p) = -kst + \ln p_\infty \qquad s = \frac{q_o}{v_q p_\infty} \tag{18}$$

where

k = a proportionality constant (ml/min)

t = time (sec)

p = specific activity of the water or suspension media at time t

v_p = volume of compartment p

q = specific activity of the animal or the inside of the skin bag at time t

v_q = volume of compartment q

p_o = value of p at $t = 0$

q_o = value of q at $t = 0$

p_∞ = value of p at $t = \infty$

q_∞ = value of q at $t = \infty$

To determine p_∞ it is assumed that at $t = \infty$ the activity will be distributed equally in the two volumes, v_p and v_q. The animals were assumed to be 79 percent water (Thorson, 1964). Knowing these volumes and the initial specific activity, the final activity at $t = \infty$ can be calculated. A plot of $\ln (p - p_\infty)$ or $\ln (p_\infty - p)$ against time then gives a straight line with a slope equal to $-ks$.

In using equations 1 and 2, certain assumptions must be made. These include: (a) The inside of the animal or skin bag and the suspension medium should behave as two well mixed compartments. The validity of these assumption will be considered in the discussion. (b) There should be no net movement of water. Ligated animals showed no net uptake of water in isosmotic sucrose solutions. Skin

bags in isomotic sucrose solutions also showed no net movement of water, after the first hour. (c) THO should act as an ideal tracer for water. The diffusion coefficient of THO is 14 percent smaller than that of H_2O^{18}, which is considered to be the most ideal tracer for H_2O (Wang et al., 1953). Thus all THO fluxes in this paper have been increased by 14 percent. (d) There should be no adverse effects due to radiation.

Statistical Treatment

Results are represented as the mean ± one standard error. The number of samples is indicated in parenthesis.

RESULTS

Osmotic Flow

Kirschner et al., (1960) have shown that the non-osmotic component (J_V^{NOS}) of the total water flow (J_V^T), is absent when sucrose bathes the epithelial side of the skin. Therefore, all measurements made in sucrose solutions will be referred to as osmotic flow (J_V^{OS}). This flow consists of a diffusion flow (J_V^D) and a hydrodynamic flow (J_V^H). In order to be able to compare the flows on isolated skin with whole animal flows, the values are expressed as flow per unit area or fluxes. The symbols for the fluxes will be J_F^D for diffusive flux, J_F^H for hydrodynamic flux and J_F^{NOS} for non-osmotic flux.

Temperature has a pronounced effect on the osmotic flux of water into intact frogs as shown by a Q_{10} of 1.9 for winter frogs (Table 1) and a Q_{10} of 2.0 to 2.8 for spring frogs as seen for the control group in Table 2. The difference between the winter and spring frogs may be due to the different seasons. De Haan and Bakker (1924) have shown that at similar temperatures the rate of water excretion is 100 percent greater in summer than in winter frogs. However, since the two groups also differed in size and age, no valid comparison between the two groups can be made concerning either season, size or age. The large temperature response for both winter and spring

Table 1. Effect of temperature on the osmotic uptake of water into intact winter frogs and across isolated skin. The epithelium in each case was bathed in 23 mM sucrose.

Preparation	5°	15°	Q_{10}
	J_F^{OS} (μl/cm^2-hr)		
Whole frog	1.40±0.27 (7)	2.62±0.75 (6)	1.9
Isolated skin	1.3 ±0.05 (8)	2.0 ±0.06 (8)	1.5

Table 2. Effect of ADH injection (50 mU/g) on the osmotic uptake of water into spring frogs at 5° C and 15° C. The external solution was 23 mM sucrose. The frogs were shaken at a frequency of 2 cycles/sec.

Preparation	5°	15°	Q_{10}
	J_F^{OS} (μl/cm^2-hr)		
Control	3.22±0.43 (5)	6.28±0.85 (5)	2.0
ADH injected	6.35±0.34 (5)	12.98±1.65 (4)	2.0

frogs is greater than can be explained by changes in viscosity which would be expected to cause only a 35 percent increase ($Q_{10} = 1.35$) in the osmotic flux. A possible interpretation of these results is that temperature is either directly or indirectly affecting the osmotic permeability (P_{OS}).

To test the direct effect of temperature on the osmotic permeability of the skin, the skin was removed and tested in vitro. The results show (Table 1) that temperature has only a slight effect on the permeability of the isolated skin. This is shown by the low Q_{10} of 1.5, very close to the value of 1.3, the expected value, due to the effects of temperature on viscosity and the osmotic gradient. Clearly, if it is the osmotic permeability that is affected, the effect is indirect.

Indirect effects of temperature on P_{OS} of skin of intact frogs could be caused by changes in the level of hormones which affect permeability or by changes in the sensitivity of the skin to these hormones. Fuhrman and Ussing (1951) have shown that the antidiuretic hormone (ADH) increases the permeability of the frog skin to water, both in vivo and in vitro. Dose responses curves done on intact animals at 5° C and 15° C are shown in Figures 6 and 7 respectively. A dose of 50 mU/g of Pitressin gives a maximum response at both temperatures, even though the magnitude of the response was lower at 5° C than at 15° C. This indicates that there is no change in the sensitivity of the skin to the hormone. Table 2 gives the values of

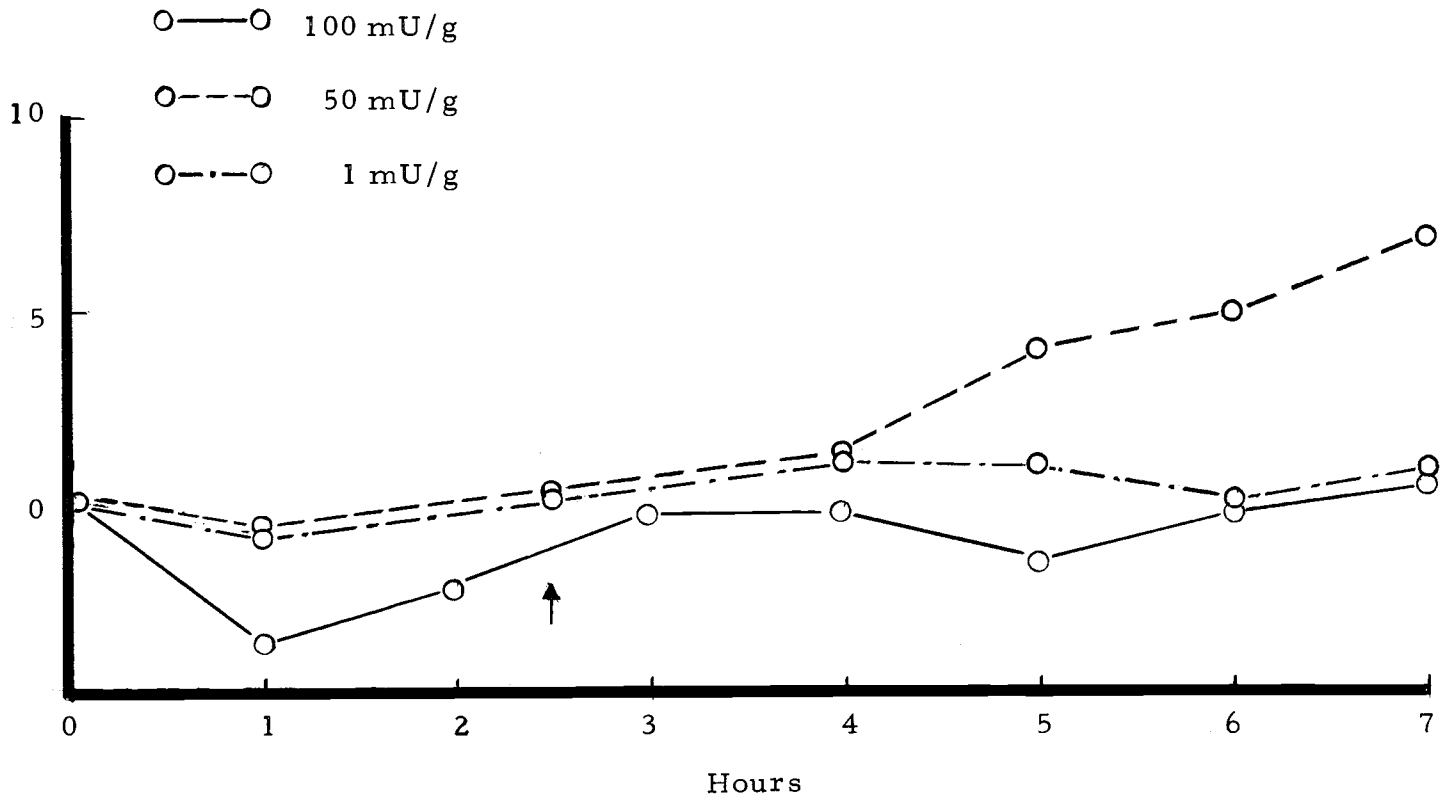


Figure 6. Dose response curve for the effect of ADH on the water movement into spring frogs at 5 ° C. The hormone injection was made at the arrow.

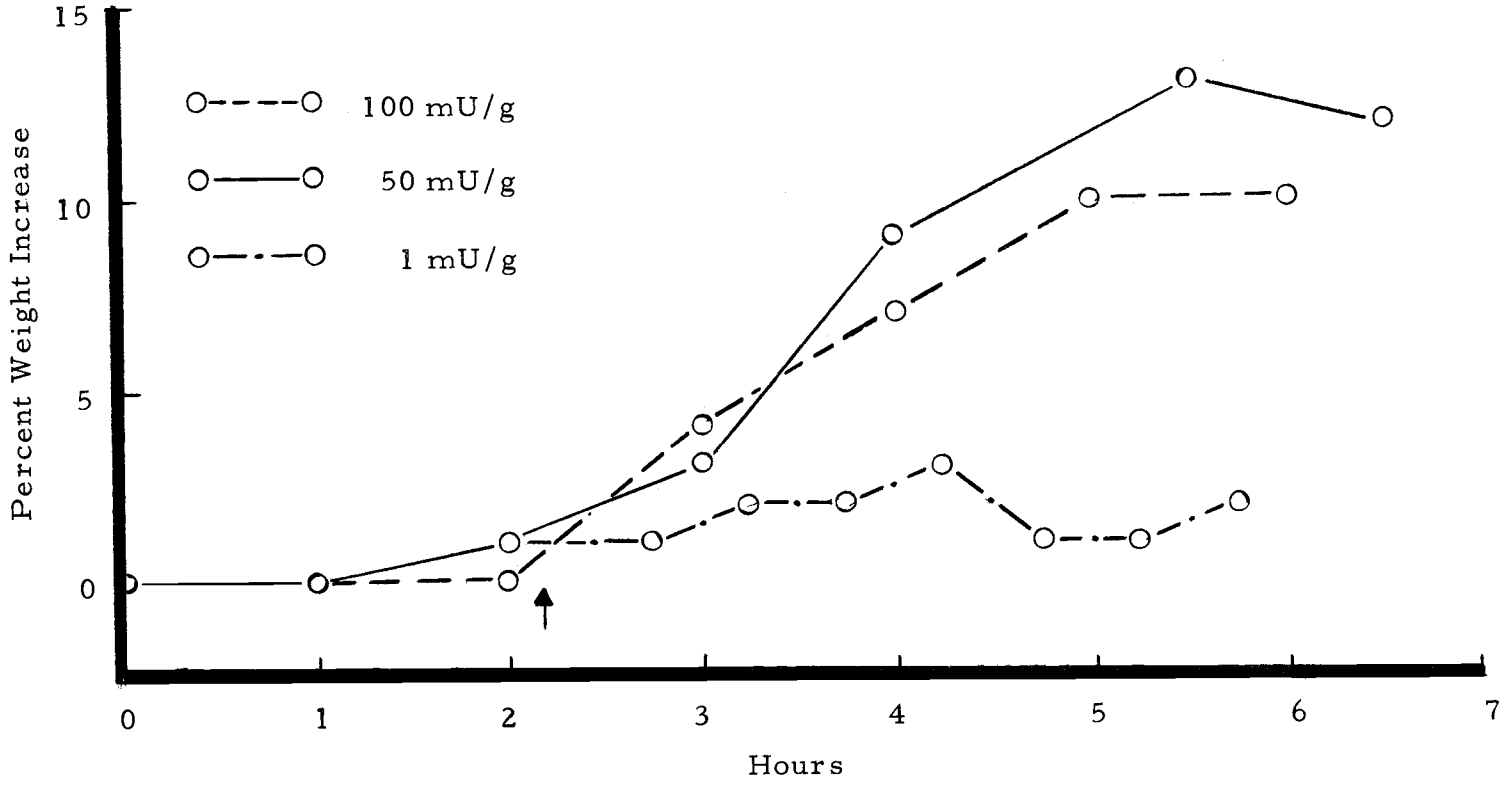


Figure 7. Dose response curve for the effect of ADH on the water movement into spring frogs at 15° C. The hormone injection was made at the arrow.

the response at 50 mU of ADH/g of frog and also for a group of controls from the same population of frogs. The Q_{10} in both cases is much greater than 1.3.

Changes in membrane permeability resulting from changes in pore size can be detected by observing changes in the ratio between the diffusive flux and the total osmotic flux (J_F^D / J_F^{OS}) as shown in equation 8. Koefoed-Johnsen and Ussing (1953) have shown that the increase in the osmotic permeability caused by ADH, can be associated with a decrease in this ratio. An increase in the reciprocal of this ratio (J_F^{OS} / J_F^D) then indicates an increase in pore size (or channel size) and thus can be used as a relative index of permeability.

Figures 8 and 9 show representative graphs from which diffusive flows were calculated according to equations 17 and 18 respectively. Table 3 shows the flows per unit area or fluxes for ADH-injected and control spring frogs. The 10^{o} rise in temperature increased the diffusion flux nearly as much as the total osmotic flux on whole animals.

The diffusive flux and the osmotic flux can be used to calculate the J_F^{OS} / J_F^D ratio. However, since the concentration gradients were not the same, the fluxes must be corrected to unit concentration difference. Thus, the fluxes were divided by their respective concentration gradients and expressed in comparable units of $\mu l/cm^2$-sec-cm H_2O. The osmotic concentration gradient was

Table 3. Effect of injection of ADH (50 mU/g) on the net diffusion (J_F^D) of water into spring frogs as measured with THO. The external solution was 225 mM sucrose.

Preparation	5°	15°	Q_{10}
	J_F^D (μl/cm^2-hr)		
Control	138±3 (7)	234±55 (6)	1.7
ADH injected	158±11 (7)	247±23 (10)	1.6

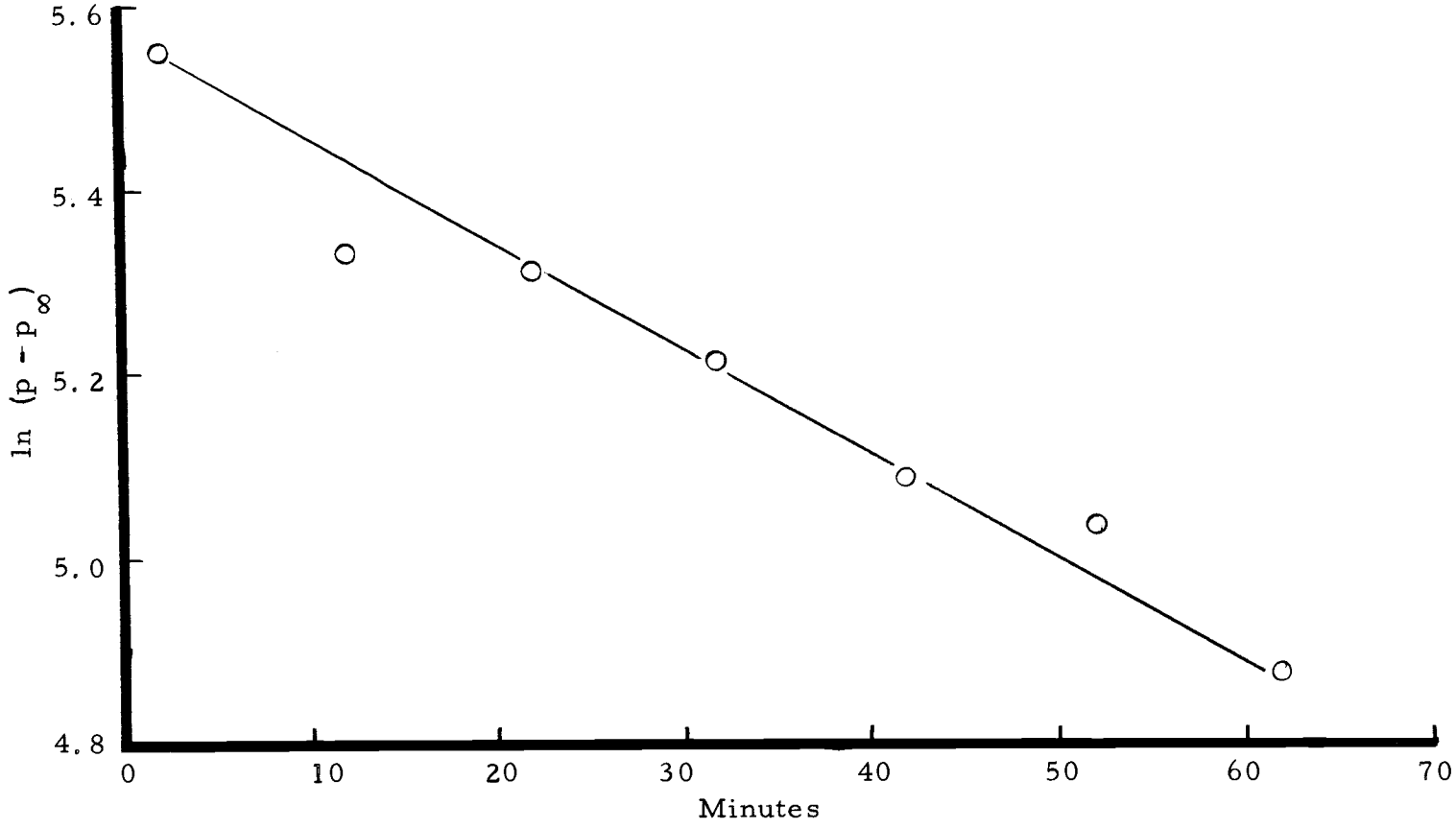


Figure 8. Representative curve of the rate of equilibration of whole frogs with THO media at 15° C.

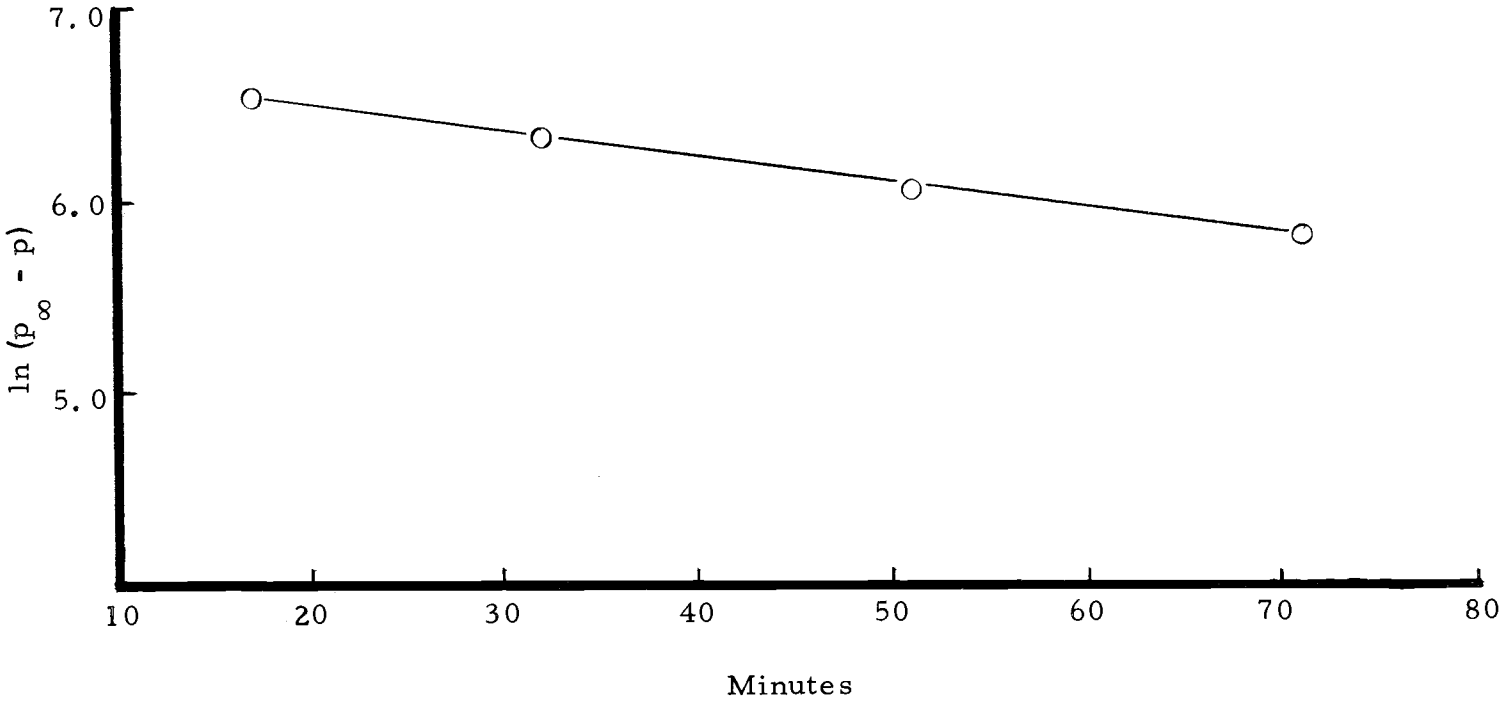


Figure 9. Representative curve of the rate of equilibration of skin bags with THO media at 15° C.

4.70×10^3 cm H_2O at 5° C and 4.86×10^3 cm H_2O at 15° C. The diffusion gradient was 1.30×10^6 cm H_2O at 5° C and 1.34×10^6 cm H_2O at 15° C. The J_F^{OS} / J_F^D ratios (Table 4) show the increase with the addition of ADH, at both 5° C and 15° C, as found by Koefoed-Johnsen and Ussing (1953). However, the ratio for the controls at 5° C is very close to the ratio at 15° C indicating that there is no change in the permeability with temperature. Diffusive values for whole frogs and isolated skin shown in Table 5 can be compared to the osmotic values in Table 1. The ratios show no permeability change in whole frogs or isolated skin with a temperature change.

These experiments indicate the existence of a diffusive flux (J_F^D) different from the osmotic flux (J_F^{OS}) in both isolated skin, as has been shown previously (Koefoed-Johnsen and Ussing, 1953; Dainty and House, 1966), and for the whole frogs. Thus, there is evidence that in whole frogs, like in isolated skin, a hydrodynamic component is present, indicating a laminar flow through pores.

Although the flux through whole animals seems to be the same as that through isolated skin, temperature has a much greater effect on the osmotic and diffusive flux in whole animals than on isolated skin. Since this difference is not due to a permeability change, and area can be assumed to remain constant, temperature must affect the concentration gradient across the external epithelia of whole

Table 4. Ratio of the osmotic flux J_F^{OS} to the diffusive flux J_F^D. The fluxes are in units of $\mu l/cm^2$-sec-cm H_2O. Spring frogs were used.

Preparation	5°	15°
	J_F^{OS} / J_F^D	
Control	$\frac{6.85 \times 10^{-4}}{1.06 \times 10^{-4}} = 6.5$	$\frac{12.9 \times 10^{-4}}{1.74 \times 10^{-4}} = 7.5$
ADH injected	$\frac{13.5 \times 10^{-4}}{1.20 \times 10^{-4}} = 11.3$	$\frac{26.6 \times 10^{-4}}{1.84 \times 10^{-4}} = 14.5$

Table 5. Diffusive movement of water across skin of intact frogs and across isolated skin as measured by THO. The epithelial surface was bathed in 225 mM sucrose. The corium of the skin bags was bathed with Ringer's solution. Winter frogs were used.

Preparation	5°	15°	Q_{10}
	J_F^D ($\mu l/cm^2$-hr)		
Whole animals	119±7.58 (6)	204±11.98 (5)	1.7
Isolated skin	119±7.54 (7)	173±16.95 (10)	1.4
	J_F^{OS} / J_F^D		
	5°*	15°*	
Whole animals	$\frac{29.8 \times 10^{-5}}{9.15 \times 10^{-5}} = 3.26$	$\frac{53.9 \times 10^{-5}}{15.4 \times 10^{-5}} = 3.5$	
Isolated skin	$\frac{27.6 \times 10^{-5}}{9.15 \times 10^{-5}} = 3.02$	$\frac{41.3 \times 10^{-5}}{12.9 \times 10^{-5}} = 3.19$	

*Calculation same as for Table 4. J_F^{OS} values obtained from Table 1.

animals.

Shaking also increased the osmotic uptake of water by about 100 percent. This can be seen by comparing the values for the spring frogs which were not shaken (Table 6) with those which were shaken (Table 2). Since this occurred both without and with ADH injection, any indirect effect on permeability due to hormone changes cannot explain the results.

The effect of temperature on the osmotic permeability of the exposed epithelia of the urodele, Ambystoma gracile, was also determined using the ratio: J_F^{OS} / J_F^{D}. This was the only method available since the skin of urodeles cannot be removed without injury because it is more firmly attached than in anurans. The values for urodeles, given in Table 7, indicate that, as in anurans, there is no change in the permeability of the exposed epithelia with a change in temperature.

Non-osmotic Water Movement

Since animals are normally in pond water which has a sufficient amount of salts to create non-osmotic water movement, the effect of temperature on this component must also be considered. The flow of water into whole animals and through isolated skin was measured in 1/10 Ringer's which is 23.0 mOsmo/l; thus, the external osmotic pressure is the same as for the measurements made in sucrose. The

Table 6. Effect of ADH injection (50 mU/g) on the osmotic uptake of water (J_F^{OS}) at 5° C and 15° C in spring frogs. The external solution was 23 mM sucrose. The animals were not shaken. Spring frogs were used.

Preparation	5°	15°	Q_{10}
	J_F^{OS} ($\mu l/cm^2$-hr)		
Control	1.01±0.07 (5)	2.85±0.32 (6)	2.8
ADH injected	2.23±0.39 (6)	7.45±0.97 (5)	3.3

Table 7. Effect of temperature on the osmotic uptake of water and the diffusive movement of THO into Ambystoma gracile. Osmotic uptake was measured in 23 mM sucrose and THO diffusion in 225 mM sucrose.

Preparation	5°	15°	Q_{10}
Osmotic uptake (J_F^{OS})	0.94±0.04 (7)	1.74±0.06 (6)	1.8
THO diffusion (J_F^{D})	103±10 (7)	233±24 (6)	2.3
	J_F^{OS}/J_F^{D}		
	$\frac{5°*}{\frac{20 \times 10^{-5}}{7.9 \times 10^{-5}}} = 2.53$	$\frac{15°*}{\frac{37.8 \times 10^{-5}}{17.4 \times 10^{-5}}} = 2.18$	

*Calculation same as for Table 4.

values of J_F^T given in Table 8 represent osmotic plus non-osmotic movement of water. Temperature has a large effect on the non-osmotic component (J_F^{NOS}) in isolated skin, but not in whole animals. Since this flow is dependent upon metabolism (Kirschner et al., 1960), temperature would be expected to have a large effect on the non-osmotic flow. Diffusion measurements through isolated skin were also made in Ringer's (Table 9) and, as seen when sucrose was present, the increase in temperature from 5° C to 15° C increases the rate of diffusion by 30 percent, a value which would be predicted from the effect of temperature on viscosity.

Table 8. Effect of temperature on the total uptake of water (J_F^T) into whole frogs and across isolated skin. The corium of the isolated skin was bathed in Ringer's solution. The epithelia in both cases was bathed in 1/10 Ringer's (23 mOsmo/l). J_F^{OS} values are from Table 1. J_F^{NOS} is calculated by subtracting J_F^{OS} from J_F^T.

Preparation	Total Flux J_F^T	Osmotic Flux J_F^{OS}	Non-Osmotic Flux J_F^{NOS}
	5° ($\mu l/cm^2$-hr)		
Whole frog	2.02±0.17 (6)	1.40±0.27 (7)	0.62
Isolated skin	1.2 ±0.1 (4)	1.3 ±0.1 (8)	-0.1
	15° ($\mu l/cm^2$-hr)		
Whole frog	3.45±0.22 (6)	2.62±0.75 (6)	0.83
Isolated skin	2.8 ±0.3 (4)	2.0 ±0.1 (8)	0.8

Table 9. Effect of temperature on the net diffusion of water across isolated skin as measured with THO. Both sides of the skin were bathed in Ringer's solution.

Preparation	5°	15°	Q_{10}
	J_F^D ($\mu l/cm^2$-hr)		
Isolated skin	160±16 (7)	202±10 (8)	1.3

DISCUSSION

Water movement through biological membranes occurs through pores. The physical dimensions of the pores cannot be measured directly but a variety of indirect estimates indicate that the radii of the pores range between 4 Å to 50 Å in different preparations (Pappenheimer, 1953; Solomon, 1968; Hays and Leaf, 1962). The rate of water movement through the pores (J_V^T) in a membrane may be expressed as the sum of a non-osmotic flow (J_V^{NOS}) and an osmotic flow (J_V^{OS}). The non-osmotic component is dependent on active transport of solute and is abolished if this transport is prevented.

In the isolated frog skin active transport of sodium drives the non-osmotic movement of water (Curran, 1960; House, 1964). This is also true of intact animals as shown in the present study. This component comprises about 25 percent of the total water movement into frogs immersed in 10 percent Ringer's solution.

Temperature does not have a marked influence on J_V^{NOS} in intact frogs ($Q_{10} = 1.3$). This suggests that the non-osmotic movement is not directly related to active solute transport for the Q_{10} for the later process is usually about 2.0 (Parsons and Alvarado, 1968; Snell and Lehman, 1957). I am not able to calculate a valid Q_{10} for J_V^{NOS} in isolated skin because this component was

abolished at 5° C. This aspect of the problem has not been pursued.

The osmotic component (J_V^{OS}) of water movement can be expressed as the sum of a diffusion component (J_V^{D}) and a hydrodynamic component (J_V^{H}). The distinction is based on the fact that the permeability constant measured with isotopic water (D_2O or THO) is lower than that obtained from measurements of osmosis for a variety of membranes including the isolated and intact frog skin (Hevesy et al., 1935; Koefoed-Johnsen and Ussing, 1953).

Temperature has a pronounced effect on the osmotic uptake of water across the intact frog skin but not the isolated skin. The activation energies (E_A) would be 6.5 Kcal/mole for J_V^{OS} and 5.1 Kcal/mol for J_V^{D} for isolated skin whereas for diffusion of water in water the E_A is 4.9 Kcal/mol (Wang et al., 1953). Comparable low values have been reported for J_V^{D} into invertebrate nerve fibers (3.5 Kcal/mole) by Nevis (1958), and human erythrocytes (3.9 Kcal/mole) by Hempling (1960) who used the data of Jacobs (1938). These data indicate that temperature does not directly affect these membranes in any way that would change the resistance to the flow of water.

Other workers, however, have found high activation energies for both osmotic water flow and THO diffusion. Hempling (1960) found a value of 9.6 Kcal/mole for osmotic flow into ascite tumor cells; Hays and Leaf (1962) found a value of 9.8 Kcal/mole for the

THO diffusion through toad bladder. These authors attribute their results to either a change in the interaction of water molecules inside the pores or to a change in pore size which results in a change in the water-membrane interaction.

The Q_{10} for osmotic flow into intact frogs was 1.9 to 2.8 (E_A = 9.6 to 15.4 Kcal/mole). In order to analyze the factors responsible for this marked effect of temperature it is advantageous to reconsider equation 1. The P_{OS} term usually incorporates ΔX, the length of the pore. By separating this term it is possible to write a similar equation:

$$J_V^{OS} = P'_{OS} \quad A_M \frac{\Delta \pi}{\Delta X} \tag{19}$$

It is doubtful (but not impossible) that A_M changes significantly with temperature. Therefore, temperature could effect either P'_{OS} or $\Delta\pi/\Delta X$, or both. It is extremely difficult to measure the local osmotic gradient $(\Delta\pi / \Delta X)$, particularly the ΔX term, so that attention was first focused on P'_{OS}. This was a logical choice because it is known that P'_{OS} in fact is a variable in the normal functioning of the skin of frogs as well as other biological membranes involved in the osmotic transfer of water. The term P'_{OS} can be further broken down into:

$$P'_{OS} = A_P \frac{K}{A_M} \tag{20}$$

As mentioned earlier, it seems unlikely that A_M is an important variable. The collective area of the pores is given by:

$$A_P = N\pi * r^2$$

where

N = number of pores, and

r = radius of each pore.

There is no evidence that N is a variable in a given membrane although this possibility cannot be dismissed completely (see Hays, 1968). If N is constant, then r^2 is the critical term determining A_P. If both diffusive and hydrodynamic flows are considered, the K term includes two variables, viscosity of the fluid (η) and the radius (r^2) of the pores in the membrane. Changes in viscosity cannot account for a Q_{10} above 1.3. Thus the only variable incorporated into the P'_{OS} term which could account for the high Q_{10} for water uptake into intact frogs is the radii of the pores (r). In fact there is a considerable body of evidence that changes in r are responsible for changes in permeability observed in many membranes (Koefoed-Johnsen and Ussing, 1953; Hays and Leaf, 1962).

Hormones derived from the hypothalamic-neurohypophysial complex of vertebrates are known to increase the water permeability of frog skin (Fuhrman and Ussing, 1951), toad urinary bladder (Hays

and Leaf, 1962), and renal tubules (Granthan and Burg, 1966). In each case there is evidence, derived by a variety of methods, that the action of these hormones is to increase the radii of pores in the membranes (Fuhrman and Ussing, 1951; Koefoed-Johnsen and Ussing, 1953; Hays and Leaf, 1962).

It is possible that temperature exerts its effect on water uptake indirectly by affecting the level of "water-balance" hormones in the blood. Injection of a massive does of ADH should swamp the system and thus minimize changes in r caused by normal fluctuations in hormone titer induced by temperature. The results do not support this hypothesis. The high Q_{10} for J_V^{OS} was not abolished by injection of 50 mU/g of ADH.

If it can be assumed that a high dose of ADH opens the pores to a maximum value and that this maximum value is not dependent on temperature then these results suggest that temperatures does not affect the radius of the pores at all. Clearly, if temperature exerted its effect by changing the radii of pores, opening the pores wide open should abolish the effect. It does not.

Further evidence that r is not the factor affected by temperature is provided by an analysis of the effect of temperature on J_V^{OS} / J_V^{D}. This can be expressed as:

$$\frac{J_V^{OS}}{J_V^{D}} = 1 + \frac{6 r \pi^*}{8 \bar{V}_{H_2O}} a_{H_2O} \qquad (21)$$

Notice that temperature and viscosity are not included in the equation and would not be expected to affect J_V^{OS}/J_V^{D} unless they had an effect on r, the radii of the pores.

Koefoed-Johnsen and Ussing (1953) have demonstrated that an increase in J_V^{OS}/J_V^{D} in toad skin probably reflects an increase in r, which affects J_V^{OS} much more than J_V^{D}. Thus if temperature exerts its effect by changing only r, one might expect a marked change in J_V^{OS}/J_V^{D}. This was not observed; both J_V^{OS} and J_V^{D} were increased by about the same factor. Notice that this does not preclude the possibility of a change in r. If in fact r were the variable responsible for the effect of temperature on J_V^{OS} then injection of ADH should decrease J_V^{OS}/J_V^{D} with an increase in temperature. The ratio is slightly increased.

What factors might increase J_V^{D}? It has already been established that r has only a slight effect. The critical factor appears to be mixing (Dainty and House, 1966).

In the studies on isolated skin, mixing was the same at both temperatures and on either side of the skin, and temperature had only a slight effect on J_V^{D}. However, in studies on intact animals it is not possible to control the degree of internal mixing. The

circulation through the skin probably is a critical factor in mixing and this undoubtedly varies with temperature. Conceivably circulation also changes J_V^H.

If circulation or internal mixing is the critical factor responsible for the effects of temperature on J_V^{OS} then it is necessary to abandon the hypothesis that P'_{OS} (equation 20) changes with temperature and focus attention on the second term, the concentration gradient, $\Delta\pi/\Delta x$. It is not possible to distinguish between effects on $\Delta\pi$ and Δx at present. But is seems reasonable to think that the collective length of the pores, Δx, could change considerably with a shift in circulation through the fine capillaries of the skin. The number of capillaries functioning at any moment in the skin is a variable dependent in part on O_2 and CO_2 tension in the medium (Poczopko, 1957). A shift in Δx would affect both J_V^D (equation 4) and J_V^H (equation 5).

Experimental evidence that circulation is important in water movement into frogs was obtained by Hevesy, Hofer and Krogh (1935). They cut the sciatic nerve to one leg of a frog and observed an increase in D_2O movement into that leg relative to the opposite leg with the nerve intact. Sectioning the nerve causes vasodilation and thus increases blood flow through the leg. Ligation of the femoral artery decreased uptake of D_2O. There is evidence that temperature affects the rate of circulation in amphibians. The Q_{10} for the heart rate is 2 to 3 in salamanders (Laurens, 1914) and frogs (personal

observations). The effects of temperature on patterns of blood flow have not been assessed.

In addition to its effects on permeability of membranes, Pitressin is also a vasoconstrictor (Woolley, 1959). Thus it might be argued that the effects of injection of this hormone into frogs, as discussed above, might reflect changes in circulation rather than permeability. However this does not appear to be the case. The effects of the hormone on water movement across isolated skin are very similar to the effect observed in intact animals. The ratio J_V^{OS} / J_V^{D} is elevated in isolated skins upon injection of ADH, because J_V^{OS} is changed without much change in J_V^{D} (Koefoed-Johnsen and Ussing, 1958). The same was true after injection of ADH into whole animals, even though circulatory changes (or mixing conditions) would be expected to affect J_V^{D} markedly (Dainty and House, 1966).

Further evidence that changes in circulation are responsible for the effect of temperature on water transfer across frog skin is provided by the effects of shaking the animals. One would not expect this to directly affect the permeability of the skin. Likewise an indirect effect caused by the release of neurohypophysial hormones seems unlikely because the effect of shaking occurred in control animals as well as ADH-treated animals. A better explanation is that the stress associated with shaking increases circulation through

the skin and thus increases water transfer.

Alvarado and Johnson (1965) have shown that the urodele, Ambystoma gracile, does not increase the permeability of the exposed epithelia after injection of arginine vasotocin (AVT, an analogue of ADH found in amphibians). However, Parsons and Alvarado (1957) have shown that the Q_{10} for the urine production is 1.8. Since these animals cannot alter their permeability by varying AVT and since they have a high Q_{10} for water uptake it seems unlikely that temperature exerts its affect via this route. The present experiments support this idea as J_V^{OS} / J_V^{D} remained constant with a temperature shift from 5° C to 15° C. These results are also what would be expected if a circulation change was causing the high Q_{10} for the movement of water into aquatic animals.

Schmidt-Nielsen and Forster (1954) have shown that at low temperatures there is a reduction in the urine flow due to a reduced filtration and that the tubular reabsorption of water, which is dependent upon Na^{+} reabsorption, was actually decreased. Thus the reduction in the osmotic uptake of water with a decrease in temperature which is characteristic of a number of fresh-water poikilotherms is adaptive. It reduces the energy expenditure required to maintain osmotic homeostasis. The energy conserved by reducing the load on the kidney can be considerable. A frog at room temperature (20° C) takes up water at a rate of 30 percent of its body weight per day and

excretes a comparable amount of dilute urine. The blood flow through the kidneys of a 100 g frog is about 90 ml/hr and the glomerular filtration rate is about 3.5 ml/hr. A substantial quantity of solute is actively absorbed from this filtrate as it passes down the tubules. For example, about 350 μmoles of Na^+ is reabsorbed each hour by a 100 g frog. This requires energy. About one mole of oxygen is required to actively transport 16-28 moles of sodium (Lassen et al., 1961; Zerahn, 1961). Using this factor it is possible to calculate that about six percent of the total metabolic rate of a 100 g frog at 20^o is required for renal sodium transport. This does not take into account other solutes which are actively transported across renal tubules or the energy required to pump blood through the kidney and provide the driving force for filtration.

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