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WATER UPTAKE BY *BUFO MELANOSTICTUS*, AS AFFECTED BY OSMOTIC GRADIENTS, VASOPRESSIN AND TEMPERATURE

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SUMMARY

1. The rate of water uptake across the skin was studied in the live toad, *Bufo melanostictus*. When toads were kept in distilled water at 29° C the uptake of water amounted to $16{\cdot}9 \pm 1{\cdot}3$ μl./cm^2/hr; when bathed in sucrose or urea solutions, the water uptake diminished with increasing osmotic pressure. There was no water uptake observed when toads were kept in 200 m-osmolar sucrose or urea.

2. Intramuscular injections of vasopressin increased the rate of water uptake from distilled water. There was a good relation between doses and responses over various time intervals. A dose of 4 m-u. vasopressin/g body wt. doubled the rate of water uptake over a period of 1 hr. The same dose of vasopressin doubled the rate of water uptake when the toads were kept in solutions of sucrose or urea of different osmolarity.

3. The rate of water uptake when the toads were bathed in sodium chloride solutions was consistently 8 μl./cm^2/hr greater than when bathed in sucrose or urea solutions of equal osmolarity. There was no water uptake when the sodium chloride solution was 285 m-osmolar.

4. Vasopressin (4 m-u./g) injected intramuscularly doubled the rate of water uptake from sodium chloride solutions of different osmolarity.

5. With solutions of potassium chloride, sodium nitrate, and potassium nitrate, in concentrations up to 150 m-osmoles/l., the rate of water uptake was found to be the same as with solutions of sodium chloride of the same osmolarity. Similarly, it was doubled by injection of vasopressin (4 m-u./g).

6. The effect of temperature on the rate of water uptake before and after injection of vasopressin was investigated in toads kept in distilled water, sucrose, or sodium chloride solutions. For temperatures between 20 and 37° C, vasopressin (4 m-u./g) reduced the activation energy involved in the process of water uptake by 4000 cal.

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7. The results agree with the view that water uptake follows a diffusion process which is facilitated by vasopressin, possibly as a result of increasing the size or number of available pores.

INTRODUCTION

Injection of mammalian neurohypophysial hormones into anuran amphibians has been found to augment the uptake of water (Brunn, 1921) to produce reabsorption of water from the bladder (Ewer, 1952), and to decrease urine formation (Sawyer, 1957). According to Bentley (1957) water uptake across the skin of the intact toad, and across the isolated toad bladder (Bentley, 1961) is proportional to the osmotic gradient, and is augmented by addition of vasopressin.

It has also been shown that the presence of vasopressin on the inside of the skin (Koefoed-Johnsen & Ussing, 1953), or on the serosal aspect of the bladder (Hays & Leaf, 1962), increases the permeability of these tissues to water and it has been suggested that the mechanism of this increased permeability is an increase in the size or the number of pores which would permit a more rapid movement of water (Sawyer, 1951). According to Huf (1936), however, vasopressin would produce an enhanced active transport of sodium which in turn would carry more water with it by 'solvent drag' (Koefoed-Johnsen & Ussing, 1953). Bourguet & Maetz (1961) have suggested that neurohypophysial hormones and their synthetic analogues exert discrete effects on sodium transport and on water permeability; and Orloff & Handler (1962) have suggested that the effect on water uptake is mediated through activation of adenosine -3′,5′-cyclic monophosphate, which has been shown by Schoessler (1964) to be concurrent with sodium transport.

The present experiments were designed to examine in *Bufo melanostictus* the responses to osmotic gradients, in the presence and absence of ions, as affected by vasopressin and by temperature.

METHODS

Animals. Adult toads (*Bufo melanostictus*) of both sexes weighing between 20 and 30 g were used. Since the experiments were conducted in Singapore, no seasonal variation was encountered. The toads were collected from fresh pond water (Na^+ 1·8–2·8 mM, K^+ 0·04–0·06 mM, Cl^- 1·6–3·0 mM) and kept with access to tap water (Na^+ 0·1–0·2 mM, K^+ 0·001 mM, Cl^- 0·1–0·2 mM). They were hydrated overnight in tap water at the same temperature which would be used during the experiment.

Experimental procedures. At the onset of the experiment the cloaca was ligatured under light anaesthesia. This was achieved by tightly tying a purse-string suture round the cloaca with three stitches, followed by a ligature with stout string of the sutured cloaca. After recovery toads were placed in 100 ml. stoppered glass jars containing 50 ml. of

either distilled water or of the solutions being investigated. The bathing solutions were replaced every hour. At intervals the toads were removed, lightly dried, and quickly weighed to within 0·05 g. When the solutions used were of high osmotic strength and were therefore expected to produce a withdrawal of water from the toads, the animals were first hydrated to + 50 % of their original weight by allowing them to rest in distilled water after ligaturing the cloaca.

Estimation of surface area of toad skin. According to Adolph (1933) the total surface of *Rana pipiens* (A) can be calculated from the formula $A = W^{2/3} \times 8$, where W = body weight in g. In the present experiments, however, the whole area of the toads was not immersed; therefore a formula expressing only the area exposed to water had to be calculated. This was done by measuring the surface of twenty-five skins from dead toads. It was found that the immersed area (A) in cm² from *Bufo melanostictus* could be expressed by the formula $A = W^{0·67} \times 6·3$, where W = body weight in g.

Calculation of water uptake. The amount of water uptake was calculated from the changes in body weight per unit time and then expressed as μl./cm²/hr.

When experiments were conducted at various temperatures, ranging from 12 to 37° C a climatic chamber was used in which temperatures were constant to within ± 1° C.

The osmolarity of solutions was calculated in accordance with data from Robinson & Stokes (1955).

Vasopressin was Pitressin (Parke Davis and Co.); it was diluted in 0·65 % NaCl solution. The volume injected varied from 0·10 to 0·30 ml./toad. All injections were given intramuscularly.

RESULTS

Effects of vasopressin on water uptake. Toads with ligatured cloaca kept in distilled water at 29° C up to 16 hr were weighed at regular intervals. There was a steady increase in their body weight due to an uptake of water. The uptake of water amounted to 16·9 ± 1·3 μl./cm²/hr (16). When vasopressin was injected intramuscularly, the amount of water taken up was markedly increased during the first half hour, and then gradually returned to normal (Fig. 1).

To see whether there was a relation between doses of vasopressin given and changes in body weight, toads kept in distilled water were injected with various amounts of vasopressin (ranging from 0·004 to 200 m-u./g) and weighed every 30 min up to 1 hr and then every hour up to 4 hr. It will be seen from Fig. 2 that a dose of 4 m-u. vasopressin/g produced an approximate 2-fold increase of body weight over the first hour following the injection: the water uptake rising from 16·9 ± 1·3 to 36·5 ± 3·6 μl./cm²/hr. This amount of vasopressin (4 m-u./g) was therefore adopted as a convenient standard dose in all subsequent experiments.

Effects of non-ionic osmotic gradients with and without injection of vasopressin. When distilled water was replaced by solutions of sucrose of various concentrations, there was a decrease in the rate of water uptake (μl./cm²/hr) which was proportional to the concentration of the outside solution. After administration of vasopressin (4 m-u./g) the rate of water uptake was doubled, but remained related to the osmotic gradient (Fig. 3). For a concentration of the bathing fluid of 200 m-osmole/l. no change in

body weight was observed. This was interpreted as the expression of an osmotic equilibrium between the toad's tissues and the outside solution. When the concentration of sucrose was increased above 200 m-osmole/l., there was a decrease in the body weight of the animals, indicating a loss of water. The rate of water loss was accentuated after intramuscular injection of vasopressin (4 m-u./g).

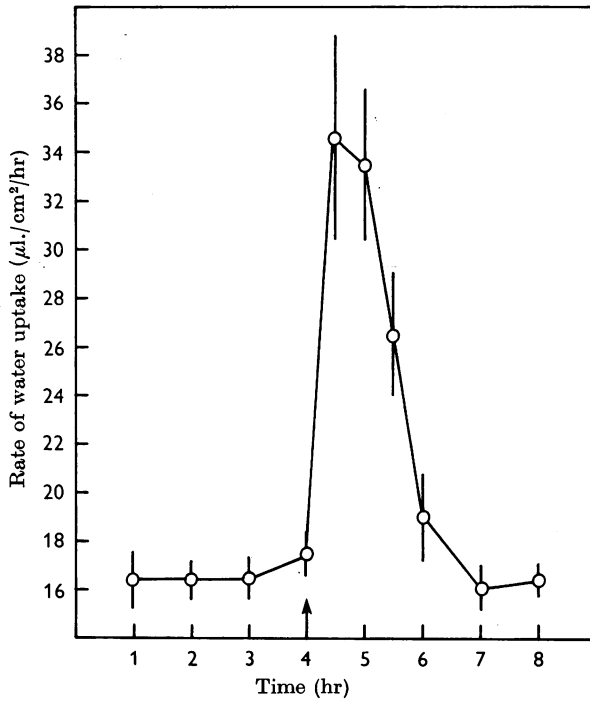

Fig. 1. Effect of vasopressin (10 m-u./g) injected intramuscularly, on rate of water uptake of toads kept in distilled water at 29° C. Each point represents the mean for five toads. Vertical lines indicate the standard error. Injection at arrow.

Similar results were observed when toads were kept in urea solutions of different concentrations. Here again, osmotic equilibrium between animals and the bathing fluid was obtained for concentrations of urea equal to 200 m-osmole/l. With higher concentrations of urea the loss of water from the toad was somewhat smaller than that observed with solutions of sucrose of similar osmolarity. The effect of injecting vasopressin (4 m-u./g) was the same as that observed when toads were kept in sucrose.

Effects of ionic osmotic gradients with and without injection of vasopressin. Toads were placed in solutions of NaCl of various concentrations, ranging from 10 to 450 m-osmole/l. For concentrations of NaCl solutions smaller than 50 m-osmole/l. the rate of increase of body weight of the animals was

greater than when kept either in similar concentrations of urea or sucrose or in distilled water. As greater concentrations of NaCl solutions were tested, there was a steady decrease in the rate of water uptake, until equilibrium was reached for NaCl solutions of 285 m-osmole/l. Since for

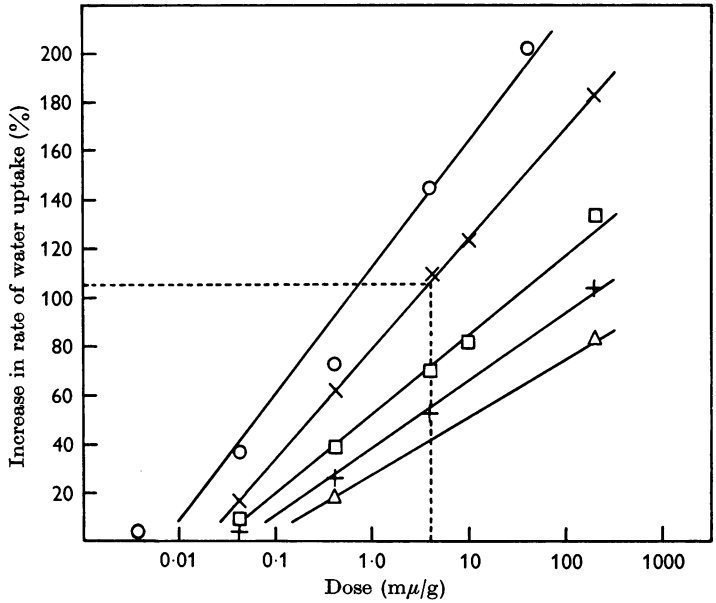

Fig. 2. Effect of vasopressin on water uptake of toads kept in distilled water at 29° C. Change in the rate of water uptake is represented on the ordinate as increase in the water uptake. Rate before injection = 16·9 μl./cm²/hr. Each point represents the average for five experiments. Doses of vasopressin are represented on a logarithmic scale on the abscissa. Responses were measured during various periods immediately after the injection; O—O 0·5 hr, ×—× 1 hr, □—□ 2 hr, +—+ 3 hr, Δ—Δ 4 hr. The dotted line represents the dose (4 m-μ./g) which caused an approximate doubling of water uptake over 1 hr.

urea and sucrose solutions osmotic equilibrium was reached for concentrations of 200 m-osmole/l. only, it could be calculated that the water uptake of toads kept in NaCl solutions was greater by 8 μl./cm²/hr, than that observed for non-ionic osmotic gradients (Fig. 4). When vasopressin (4 m-u./g) was injected intramuscularly, the rate of water uptake was roughly doubled though the position of equilibrium remained unaffected at 285 m-osmole/l. For NaCl solutions between 285 and 450 m-osmole/l. there was a decrease of body weight due to loss of water through the toad skin, which was further enhanced after injection of vasopressin.

In an attempt to see whether the effects were due to either Na^+ or Cl^- ions, experiments were done using KCl, $NaNO_3$ and KNO_3 solutions of different concentrations. Using solutions of each one of these salts in

concentrations ranging from 0 to 130 m-osmole/l. the rates of water uptake were closely similar to those observed with NaCl solutions, and were similarly doubled after injection of vasopressin (4 m-u./g).

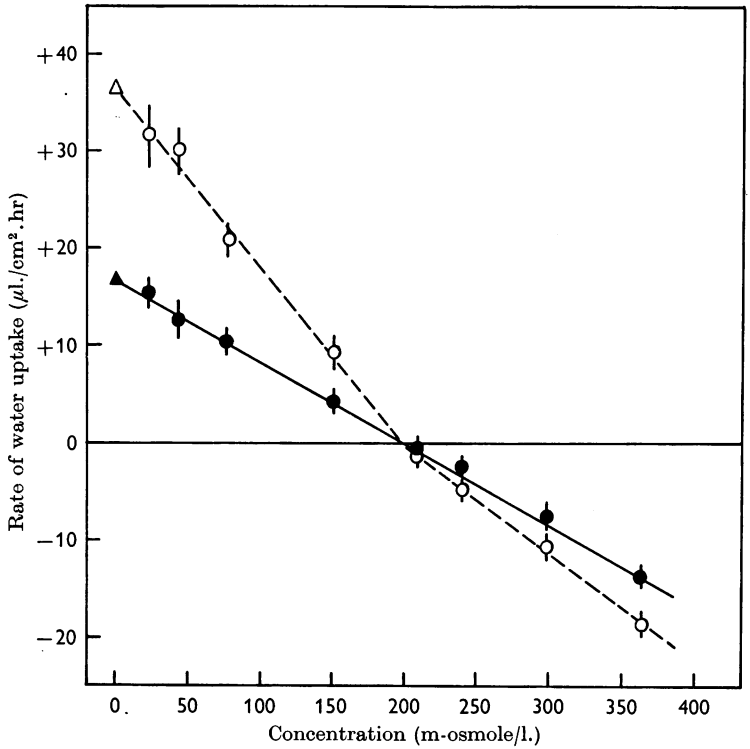

Fig. 3. Relation of rate of water uptake of toads to osmolarity of sucrose solutions at 29° C. ▲ rate of water uptake (μl./cm².hr) before, and Δ after injection of vasopressin (4 m-μ./g) when toads were kept in distilled water; ●—● rate of water uptake (μl./cm².hr) before, and ○-- -○ after injection of vasopressin (4 m-μ./g) when toads were kept in sucrose solutions. Osmolarity of sucrose solutions is represented on the abscissa. Each point represents the mean of six observations. Vertical lines indicate the standard error.

Effect of temperature on rates of water uptake. Toads were kept either in water, or in solutions of either sucrose or of NaCl of different concentrations at temperatures ranging from 12 to 37° C. Values for water uptake obtained from changes in their body weight were estimated before and after intramuscular injections of a standard dose of vasopressin. For all concentrations of solutions used, whether or not the animals had been injected with vasopressin, water uptake increased with rise in temperature; this, however, did not affect the osmotic equilibrium which as in previous experiments remained at 200 m-osmole/l. for sucrose and 285 m-osmole/l. for NaCl (Table 1).

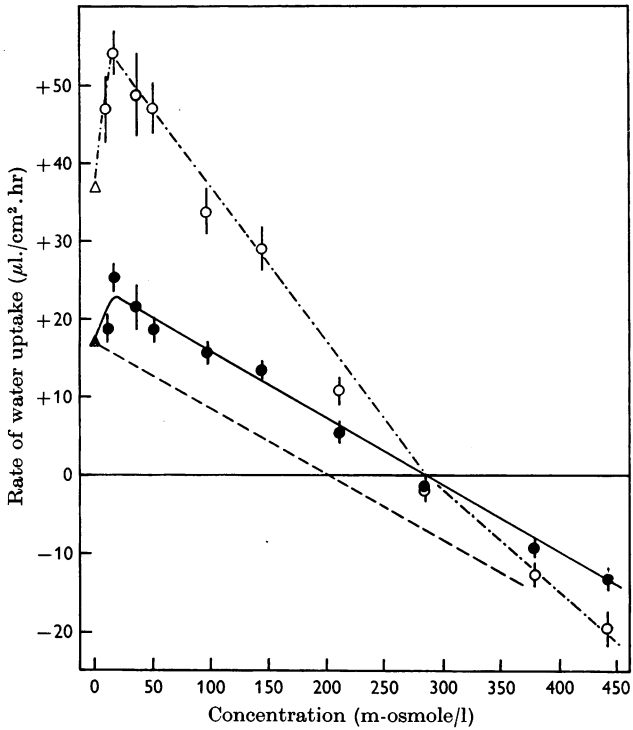

Fig. 4. Relation of rate of water uptake of toads to osmolarity of sodium chloride solutions at 29° C. ▲ rate of water uptake (μl./cm².hr) before, and Δ after injection of vasopressin (4 m-u./g) when toads were kept in distilled water; ●—● rate of water uptake (μl./cm².hr) before, and ○-.-.-.-○ after injection of vasopressin (4 m-u./g) when toads were kept in NaCl solutions. Osmolarity of NaCl solution is represented on the abscissa. Each point represents the mean of six observations. Vertical lines indicate the standard error. (Dotted line shows the curve for sucrose, as represented in Fig. 3).

TABLE 1. Rate of water uptake (μl./cm²/hr) from solutions of different osmolarity at various temperatures. Each value is the mean of five experiments, with its S.E.

		37° C	33° C	29° C	24° C	20° C	12° C
Distilled water	Before vasopressin	34·5 ± 3·3	24·7 ± 1·1	17·0 ± 1·3	14·0 ± 0·8	10·5 ± 0·5	6·4 ± 1·5
	After vasopressin	54·0 ± 5·5	41·3 ± 2·6	36·5 ± 3·6	30·5 ± 2·6	25·5 ± 3·8	10·3 ± 1·9
Sucrose 73 m-osmole/l.	Before vasopressin	17·6 ± 0·9	13·2 ± 0·8	12·4 ± 0·9	8·3 ± 1·2	6·5 ± 1·3	—
	After vasopressin	32·6 ± 2·4	20·4 ± 0·6	21·8 ± 4·6	18·0 ± 2·6	15·0 ± 2·0	
NaCl 51 m-osmole/l.	Before vasopressin	38·0 ± 3·1	28·1 ± 2·1	20·5 ± 1·7	19·3 ± 1·8	—	—
	After vasopressin	55·4 ± 5·3	—	47·4 ± 4·1	40·5 ± 2·5		
NaCl 97 m-osmole/l.	Before vasopressin	32·6 ± 4·0	23·3 ± 0·9	15·5 ± 1·1	14·5 ± 0·7	11·3 ± 1·3	10·3 ± 4·0
	After vasopressin	46·8 ± 3·9	—	33·7 ± 3·1	32·9 ± 4·1	30·7 ± 2·7	14·7 ± 1·1
NaCl 191 m-osmole/l.	Before vasopressin	14·8 ± 1·9	11·3 ± 1·0	8·0 ± 0·8	8·6 ± 1·0	6·1 ± 0·2	4·5 ± 0·5
	After vasopressin	18·1 ± 1·5	—	19·7 ± 2·6	12·6 ± 1·0	10·7 ± 1·7	5·6 ± 0·6
NaCl 285 m-osmole/l.	Before vasopressin	−4·0 ± 1·0	−6·0 ± 1·5	−2·1 ± 0·8	−1·7 ± 0·2	—	—
	After vasopressin	−8·1 ± 1·0	—	−2·0 ± 1·0	−2·7 ± 0·3		

It is well known that kinetic energy of molecules, as represented by their temperature, is the most obvious source of energy needed for activation (Bayliss, 1959). It was therefore of interest to see whether there was

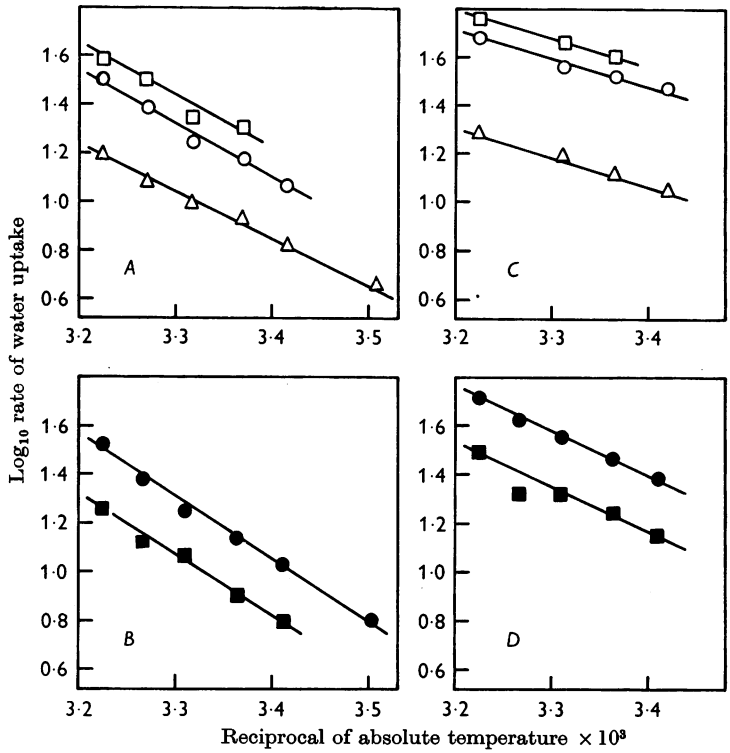

Fig. 5. Arrhenius plots of logarithm of water uptake (μl./cm²/hr) for toads kept in various solutions at temperatures between 12 and 37° C. *A* and *B*, before vasopressin; *C* and *D*, after vasopressin (4 m-u./g). Data from Table 1. ●—● water, ■—■ 73 m-osmolar sucrose; □—□ 51 m-osmolar NaCl, ○—○ 97 m-osmolarNaCl, Δ—Δ 191 m-osmolar NaCl.

TABLE 2. Values of activation energy derived from Arrhenius equation obtained from Fig. 5

Bathing solution	Treatment	Concentration (m-osmole/l.)	Calculated slope		Estimated activation energy (cal.)
Water	—	—	2·56		12,000
Water	Vasopressin	—	1·75		8,000
Sucrose solution	—	73	2·50		12,000
Sucrose solution	Vasopressin	73	1·85		8,000
NaCl solution	—	191	1·88		
		97	2·26	2·06	9,000
		51	2·04		
NaCl solution	Vasopressin	191	1·15		
		97	1·03	1·08	5,000
		51	0·98		

All injections of vasopressin were 4 m-u./g.

a relation between the rate of water uptake (μl./cm^2/hr) and the temperature. It will be seen from Fig. 5 that there was a linear relation between water uptake and temperature which could be expressed by Arrhenius equation (Bayliss, 1959). From the slopes of the different lines so obtained the activation energy for each set of conditions could be calculated. Table 2 shows that in the range of temperatures used the activation energy necessary for water uptake was of the same order of magnitude when the toads were kept in water or in a solution of sucrose, but was smaller when the bathing solution contained NaCl. Of interest was the observation that whether the toads were kept in water, in a non-ionic or in an ionic solution, injection of a standard dose of vasopressin (4 m-u./g) reduced the activation energy necessary for water uptake by a similar amount.

DISCUSSION

While it has been frequently postulated that neurohypophysial hormones augment water entry through the skin of amphibians by increasing the size of pores, there have been divergent opinions as to the relative importance of osmotic gradient and sodium transport in this movement. From the present results it would appear that when non-ionic solutes are used in the bathing fluid, the rate of water entry across the skin of the toad is directly related to the osmotic gradient. This is true for temperatures between 12 and 37° C used in this investigation. It would also appear that when the animals have been injected with vasopressin at a dose of 4 m-u./g (0·01 n-mole/g) the amount of water that goes through the skin is enhanced but is still directly related to the osmotic gradient. This too is true for temperatures between 12 and 37° C.

The activation energy for self-diffusion of water is 5000 cal. (Wang, 1965). Since the activation energy for entry of water from non-ionic solution is now found to be of the order of 12,000 cal, the movement of water across toad skin cannot be regarded as being 'diffusion-limited'. We do not visualize that every water molecule which collides with the skin succeeds in penetrating it, but rather that only molecules at a higher energy level will be able to do so. The process may be considered to be one of diffusion through pores, and to be 'membrane-limited'. The action of vasopressin in reducing the activation energy from 12,000 to 8000 cal would therefore agree with the hypothesis that the hormone increases either the size or the number of pores through which water enters.

When sodium chloride was present on the outside of the skin, the rate of fluid uptake was always greater than from non-ionic solutions of equivalent osmolarity. At concentrations above 50 m-osmole/l. at 29° C the rate of fluid uptake from solutions which provided sodium chloride was consistently 8 μl./cm^2/hr greater than from non-ionic solutions of equivalent

osmolarity. A similar finding has been reported by Bentley (1957) in *Bufo marinus*. According to Bentley, however, an equilibrium position was reached when the bathing fluid had an osmotic activity equivalent to 333 m-osmole/l. In the present investigation on *B. melanostictus*, equilibrium was reached with the non-ionic bathing solutions at 200 m-osmole/l. and with ionic solutions at 285 m-osmole/l. The 200 m-osmolar non-ionic solution may be thought to be in equilibrium across the skin with the extracellular fluid of the toad, which by inference will be exerting the same osmotic pressure. When, therefore, 285 m-osmolar sodium chloride solution is found to be in equilibrium, it would seem to indicate that the active ion transport mechanism of the skin is carrying ions inwards, and the water following these ions gives the effect of water movement against an osmotic gradient. A similar phenomenon has been reported by Diamond (1962) who showed that the gall bladder of fresh water fishes transported Na^+ and Cl^- and produced an effective water movement against an osmotic gradient of 40 m-osmoles. Since the temperature of his experiments was not quoted it is not possible to compare directly his value of 40 with the present finding of 85 for the osmotic gradient.

Further indication of the significance of sodium transport in the augmented fluid uptake from sodium chloride solution may be found in consideration of the effect of temperature. The activation energy of 9000 cal found for fluid uptake from sodium chloride solutions was significantly the same for different osmolarities, and was consistently 3000 cal less than the activation energy for uptake from non-ionic solution. If we seek to correlate this reduction with the activation energy for Na^+ transport agreement is at least possible. The activation energy of Na^+ transport in amphibia has been estimated as 1300–2900 cal/mole Na^+ (Zerahn, 1956) and 6200 cal/mole Na^+ (Zerahn, 1961)—that is from 2000–6000 cal/mole of Na^+ transported. Na^+ carries a shell of water molecules with it, the number having been variously estimated as from 4 (Hasted, Ritson & Collie, 1948) to 0·7–1·2 (Robinson & Stokes, 1955). If an activation energy of 6000 cal/mole of Na^+ is accepted for the sodium transport system, and if it is assumed that each Na^+ carries with it 2 molecules of H_2O, then the expected activation energy for this water movement will be 3000 cal/mole of H_2O. This coincides with the 3000 cal/mole difference between ionic and non-ionic solutions which has been observed in the present investigation. If, on the other hand, we accept the older estimate of 2000 cal/mole of Na^+ for the activation energy of the sodium transport system, and assume that each Na^+ carries with it 4 molecules of H_2O, then the expected activation energy for water movement accompanying sodium will be 500 cal/mole of H_2O. All that can be said at present, is that the lowering of activation energy for water movement when the toad is bathed in saline solution

may correspond to the activation energy of sodium transport and may indicate that water which forms the shell of hydration round the sodium ion is being carried across the membrane attached to that ion.

The effect of vasopressin in reducing the activation energy when sodium chloride is present from 9000 to 5000 cal, is interesting chiefly in relation to the previous findings. Whether the skin was bathed in non-ionic solution, in which case activation energy was 12,000 cal, or in ionic solutions, in which case it was 9000 cal, the effect of vasopressin 4 m-u./g was to decrease the activation energy by 4000 cal. This would appear to support the suggestion made by Koefoed-Johnsen & Ussing (1953) that vasopressin alters the permeability of epithelial cells and thus varies a rate-limiting part of the total transepithelial movement. According to Ussing (1964) the transport of sodium ions, which has been generally supposed to operate on the serosal aspect of the epithelial cells is limited by the size of the pores of the cell membrane and the 'cloud' of water molecules adherent to the ion. If this is so, an increase in pore size brought about by vasopressin would permit easier transport of sodium ion in the same way that it permits easier diffusion of water. This is supported by the present results.

The possibility that K^+ and NO_3^- can replace Na^+ and Cl^- is of interest. As there is such a similarity between the rate of water uptake from solutions providing Na^+ or K^+ and Cl^- or NO_3^-, it would seem reasonable to suppose that they are in some way capable of replacing each other. If the sodium pump operates on the serosal aspect of the epithelium, then the normal process in the presence of sodium chloride would be that sodium chloride would be pumped out of the epithelial cells into the extracellular fluid accompanied by water, and sodium chloride and water would then diffuse into the cells from the bathing fluid. In the presence of potassium chloride, sodium nitrate or potassium nitrate, the pump would operate on the serosal aspect as usual, and sodium chloride and water would move out into the extracellular fluid, whereupon water and the solute would diffuse into the cells. The pattern of active transport of sodium ion on the serosal aspect of epithelial cells is analogous to the active transport proposed by Whittembury, Sugino & Solomon (1961) on the serosal aspect of *Necturus* tubule cells, and it is interesting to note that these workers suggested a replacement process, similar to that now proposed.

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