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THE ACTION OF NEUROHYPOPHYSIAL HORMONES ON THE WATER AND SODIUM METABOLISM OF URODELE AMPHIBIANS

BY P. J. BENTLEY AND H. HELLER
From the Department of Pharmacology, University of Bristol

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When anuran amphibians (frogs and toads), kept in water, are injected with neurohypophysial extracts, the body weight increases for several hours ('water balance' or 'Brunn' effect). The magnitude of the effect depends not only on the dose and the neurohypophysial peptide used but apparently also on the habitat of the species. While, for example, no gain in weight has been found in the aquatic *Xenopus laevis* (Ewer, 1952) the body weight of another toad, *Bufo americanus*, which is well adapted to terrestrial life, may rise by as much as 45% (Steggerda, 1937).

The water balance effect in frogs and toads has been shown to be the result of an increase of water absorption through the skin and a decrease in the rate of urine flow. Absorption of water from the anuran urinary bladder is also increased by hormones of the neurohypophysis. The response of these organs to the various active peptides that occur in the vertebrate neurohypophysis may show a marked specificity: for example, in many species of frogs oxytocin has been reported to be much more effective than vasopressin, but in toads, arginine vasopressin seems usually to be the more potent peptide (see Landgrebe, Ketterer & Waring, 1955).

Urodeles have so far been little investigated. Bělehrádek & Huxley (1927), Dow & Zuckerman (1939), Jørgensen, Levi & Ussing (1946) and Schreiber & Schreiber (1950) found that axolotls responded to large doses of commercial preparations of mammalian posterior pituitary hormones with increases of body weight of up to 7%, and Steggerda (1937) has reported that an injection of Pituitrin raised the body weight of *Necturus* by about 4%.

Neurohypophysial hormones have also been shown to influence the sodium metabolism of amphibians. They increase active sodium transport across the skin and urinary bladder in anurans (Ussing & Zerahn, 1951; Morel, Maetz & Lucarain, 1958; Leaf, Anderson & Page, 1958), and the renal excretion of an injected load of sodium is facilitated (Boyd & Whyte, 1939; Sawyer & Sawyer, 1952). In the larval urodele *Ambystoma* Jørgensen *et al.* (1946) found that injections of the pressor fraction of

posterior pituitary extracts induced a net uptake of sodium in animals kept in dilute Ringer's solution.

The aims of the present investigation were threefold. First, to determine the response of urodeles with different habitats (*Triturus*, *Ambystoma* and *Necturus*) to the pure or highly purified neurohypophysial hormones now available. Experiments with 8-arginine oxytocin (vasotocin) seemed of particular interest since this peptide has been found in the neural lobe of various species of frogs and toads (Pickering & Heller, 1959; Sawyer, Munsick & van Dyke, 1959; Acher, Chauvet, Lenci, Morel & Maetz, 1960; Follett & Heller, 1963) and has recently (B. K. Follett & H. Heller, unpublished results) also been pharmacologically characterized in urodeles. Secondly, to analyse the mechanism of the water retention produced by these hormones in urodeles, and thirdly, to investigate the effect of neurohypophysial hormones on the metabolism of sodium in this subclass of the Amphibia.

METHODS

Animals. Adult newts (*Triturus alpestris* and *Triturus cristatus*) of both sexes were kept in glass tanks on moistened moss and gravel at 18–22° C. The axolotls (*Ambystoma tigrinum*) used were also of both sexes. Larval animals were kept in a glass tank containing well-aerated tap water at 18–20° C. Metamorphosed axolotls were kept in a terrarium with free access to water. Adult mud puppies (*Necturus maculosus*), again of both sexes, were kept in dechlorinated and aerated tap water in glass tanks.

Experimental procedures

Intact animals. Each newt or metamorphosed axolotl was placed into a 2 l. beaker containing tap water to a depth of 1 in. (sodium concentration: 0·38 m-mole/l.). Larval axolotls and mud puppies were placed singly into large glass vessels containing 2 l. of tap water. Water temperature varied from 19 to 22° C. Axolotls and newts were placed into the receptacles on the afternoon before the day of the experiment. The mud puppies were only transferred 3–4 hr before an experiment started. All animals were carefully dried with a soft cloth and were weighed to 0·05 g at hourly intervals on an automatic balance. In order to determine the effect of a given dose of hormone, weighing was continued until the mean weight of the animals showed a decline.

In some experiments newts received an intraperitoneal injection of 0·1 or 0·2 ml. of a 1 % (wt./vol.) solution of phenol red. For urine collection the animals were fitted with a thin-walled rubber finger stall which was tied with a double silk ligature rostral to the cloacal opening. In order to test the tightness of the ligature and to ensure that the 'bag' was fitted to the animals when their urinary bladder was empty, the newts were injected with phenol red and the bag tied on only after urine (coloured by the dye) had been spontaneously voided. Animals in which a leak was detected were rejected. In similar experiments on *Necturus* the urine was collected in rubber condomes.

One group of experiments on *Triturus* was so designed that contact of the newts' skin with the excreted urine was avoided: one finger stall was tied about 2 mm caudal to the cloacal opening and another was slipped over the first and tied rostral to the cloaca. Thus the urine was voided into the interspace between the two finger stalls. Control experiments (comparisons of the urinary volume between animals with a single and animals with a double bag) showed that urine was passed freely. At the beginning of experiments in which urine collecting bags were used the animals were weighed, injections made, the bag or bags

fitted and the animals re-weighed. At the end of the experiments the animals were weighed, the bag removed, tied and the animal re-weighed. The bags were weighed to the nearest milligramme on an automatic balance, the urine was then removed for analysis and the bags dried and re-weighed.

These procedures made it possible to calculate the following parameters:

$$\text{Water uptake (g/100 g body weight)} = \frac{(W_E - W_B) \times 100}{W_0}. \quad (1)$$

$$\text{Water retention (g/100 g body weight)} = \frac{(W_S - W_0) \times 100}{W_0}. \quad (2)$$

$$\text{Urine volume (ml./100 g body weight)} = \frac{(W_E - W_B) - (W_S - W_0) \times 100}{W_0}, \quad (3a)$$

or

$$\frac{(B_U - B_L) \times 100}{W_0}, \quad (3b)$$

where

W_0 = weight of animal at beginning of experiment;
W_S = weight of animal at end of experiment;
W_B = weight of animal with urine collecting bag attached, at beginning of experiment;
W_E = weight of animal with urine collecting bag attached, at end of experiment;
B_L = weight of empty bag;
B_U = weight of bag with urine.

The figures for urine volume in Tables 1, 2 and 4 were calculated by means of equation (3*a*). The urine volumes were also calculated from equation (3*b*) and similar results were obtained. However, because of the greater accuracy of the experimental procedures involved, results based on equation (3*a*) were preferred.

Measurements of net sodium transfer in vivo. The animals were starved for a week before the experiment. The urinary bladder was emptied by catheterization; an injection of the hormone or control solution was given and the newt placed in a beaker with 200 ml. tap water whose sodium concentration had been accurately determined. *Necturus* were placed in a beaker containing 700 ml. of tap water. After 4 or 5 hr the bladder was again emptied but this time into the fluid in which the animal had been sitting. The sodium concentration of this fluid was determined.

Estimation of sodium. An EEL (Evans Electroselenium) flame photometer was used.

Injections of neurohypophysial hormones. Hormone solutions were suitably diluted with 0·6 % NaCl solution which was also used for control injections. All injections were given intraperitoneally with a tuberculin or a Hamilton (0·05 ml.) micro-syringe and a 214R (Agla) or a Hamilton needle. Doses were calculated per gram body weight. The volume of fluid injected did not exceed 0·5 ml./100 g body weight.

Measurement of water and sodium transfer in isolated organs. The methods used were similar to those described by Bentley (1958, 1960) for the isolated urinary bladder of the toad.

Isolated skin. The animal was killed, a piece of skin stripped off and tied in the form of a bag to the end of a short piece of glass tubing with the dermis facing outwards. The bag was immersed in a test-tube containing 30 ml. of Ringer's solution buffered with bicarbonate (pH = 7·8). The Ringer's solution was aerated and kept at 22° C. For measurements of the rate of water transfer the skin bag was filled with dilute (1:10) Ringer's solution, i.e. the osmotic gradient was 220 m-osmole/l. Undiluted Ringer's solution was used when measuring potential differences (p.d.) and the short-circuit current (SCC). Net flux of water was measured by weighing the glass tubing together with the attached bag on an automatic balance to 1 mg before and after incubation for 1 hr in Ringer's solution; the

bag was then placed in fresh Ringer's solution containing the hormone and re-weighed after a further hour. The SCC and p.d. were measured as described by Bentley (1960), using KCl–agar bridges and calomel cells connected to a potentiometer and battery from which current was tapped to short-circuit the preparation as required. After the experiment the surface area of the skin was measured by drawing its outline on squared paper.

Isolated urinary bladder. The bladder was carefully removed, tied to the end of a glass tube and treated in the same way and exposed to the same solutions as the skin bags.

In both the isolated skin and the isolated bladder preparations the hormones were added to the fluid bathing the dermal or serosal surface respectively.

Hormone preparations. The following were used: synthetic oxytocin (Syntocinon, Sandoz), arginine vasotocin (prepared from pollack pituitaries according to Heller & Pickering, 1961); arginine vasopressin (prepared from ox pituitary posterior lobes according to Sachs (1959) or by an unpublished method of B. K. Follett & H. Heller); lysine vasopressin, either synthetic (Sandoz) or prepared from pig pituitary posterior lobes according to B. K. Follett & H. Heller.

Vasotocin and the vasopressins were assayed on the blood pressure of anaesthetized rats by Dekanski's (1952) modification of Landgrebe, Macaulay & Waring's (1946) method. The 3rd International Standard Powder was used as reference.

The structural formulae of these neurohypophysial hormones have now been verified by synthesis. It seemed therefore appropriate to express doses as moles of pure peptide even though potency estimations of the pure hormones differ from laboratory to laboratory. This is unavoidable in view of the multiplicity of assay methods in use and the differences in their execution. Moreover, all these methods have large errors. The results obtained in different laboratories have therefore been collated. Oxytocin: potency estimations vary from 400 to 450 u./mg (du Vigneaud, Ressler, Swan, Roberts & Katsoyannis, 1954; Boissonnas, Guttmann, Berde & Konzett, 1961); 450 u./mg (453 m-u./μ-mole) was adopted. Arginine vasotocin: potency estimates for the synthetic peptide vary from 124 to 245 pressor u./mg (Katsoyannis & du Vigneaud, 1958; Berde, Huguinin & Stürmer, 1962). When synthetic 8-arginine oxytocin became available it assayed at 232 (pressor) u./mg (244 m-u./μ-mole) and this value was adopted. Arginine vasopressin: the range of potencies is 350–450 u./mg (du Vigneaud, Gish, Katsoyannis & Hess, 1958; van Dyke, 1959; Light, Studer & du Vigneaud, 1959; Boissonnas *et al.* 1961) and 400 u./mg (434 m-u./μ-mole) was used. Lysine vasopressin: du Vigneaud, Bartlett & Jöhl (1957) and Boissonnas & Guttmann (1960) obtained almost identical results, namely 268 to 280 u./mg; 272 m-u./mg (285 m-u./μ-mole) was used.

RESULTS

Effect of 8-*arginine oxytocin* (*vasotocin*) *on water retention by* Ambystoma, Necturus *and* Triturus

Figures 1 and 2 show the effect of large doses of vasotocin on three species of urodele amphibians, the larval axolotl (*Ambystoma tigrinum*) (Fig. 1*a*), the neotenous mud puppy (*Necturus maculosus*) (Fig. 1*b*) a wholly aquatic species, and a newt (*Triturus alpestris*) (Fig. 2) which lives mainly on land. The effects shown were probably maximal. Higher doses (116 μ-mole/kg in *Ambystoma* and 333 μ-mole/kg in *Triturus*) failed to produce greater increases in body weight ($P > 0{\cdot}8$ and $> 0{\cdot}8$). However, since the response of amphibians to neurohypophysial hormones is influenced by the season of the year (Heller, 1930) greater responses may have been missed. Weight increases of 20% and more were encountered

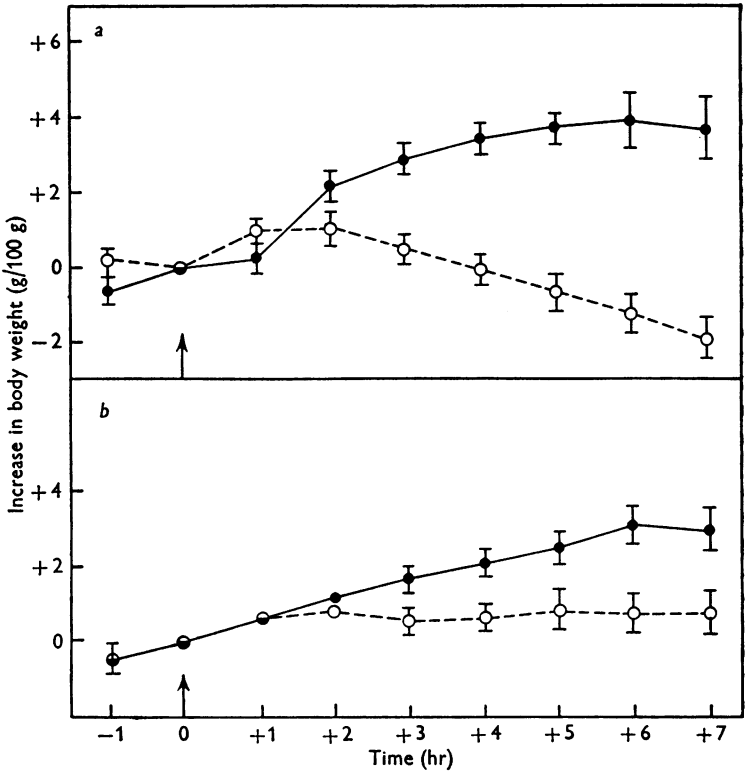

Fig. 1. *a*, Effect of arginine vasotocin on water retention (= increase in body weight) by larval *Amblystoma* kept in water. ●——● Animals injected with vasotocin (36 μ-mole/kg); ○——○ control animals injected with 0·6 % NaCl solution. Each point represents the mean value for nine axolotls. *b*, Effect of arginine vasotocin on water retention (increase in body weight) by *Necturus* kept in water. ●——● animals injected with vasotocin (112 μ-mole/kg); ○——○ control animals injected with 0·6 % NaCl solution. Each point represents the mean for twelve mud puppies. Injections at arrow. The vertical bars indicate the standard error.

in individual newts. The weight increases in *Necturus* were small but significant ($P < 0{\cdot}01$).

Dose–response relation of naturally occurring neurohypophysial hormones in Triturus *and* Ambystoma

Older reports (see Heller, 1945) suggested that frogs and toads differ in their quantitative response to oxytocin and vasopressin. Vasotocin seems to be the most active of the identified neurohypophysial hormones in these groups of anurans (H. Heller & P. J. Bentley, unpublished). The effects on water retention in *Triturus* and *Ambystoma* of arginine vasotocin,

arginine vasopressin, lysine vasopressin and oxytocin were compared in the present investigation.

The responses in *Triturus* (Fig. 3) indicate that in this family of urodeles vasotocin is the most potent of the active peptides tested. There was good discrimination between vasotocin and the vasopressins, and between lysine

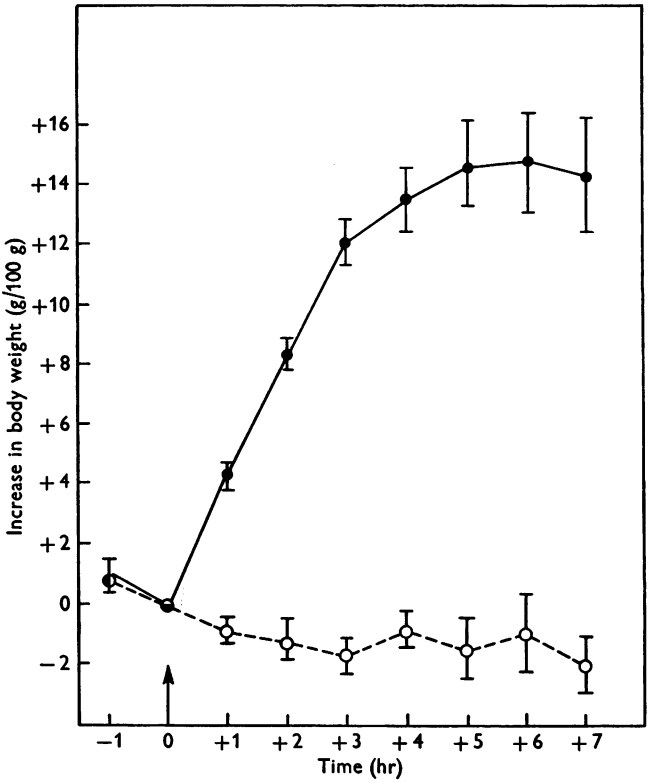

Fig. 2. Effect of arginine vasotocin on water retention (= increase in body weight) by *Triturus alpestris* kept in water. ●——● animals injected with vasotocin (36 μ-mole/kg); ○——○ control animals injected with 0·6% NaCl solution. Each point represents the mean for nine newts. Injections at arrow. The vertical bars indicate the standard error.

and arginine vasopressin respectively. The action of oxytocin was about 40 times weaker than that of vasotocin.

Figure 4 shows that in larval *Ambystoma* vasotocin and arginine vasopressin gave much the same response. Lysine vasopressin was about 20 times weaker than either. Note, however, that the sensitivity of the axolotl for the hormones was much greater than that of *Triturus*. Maximal weight increases were obtained in *Ambystoma* with doses of vasotocin and

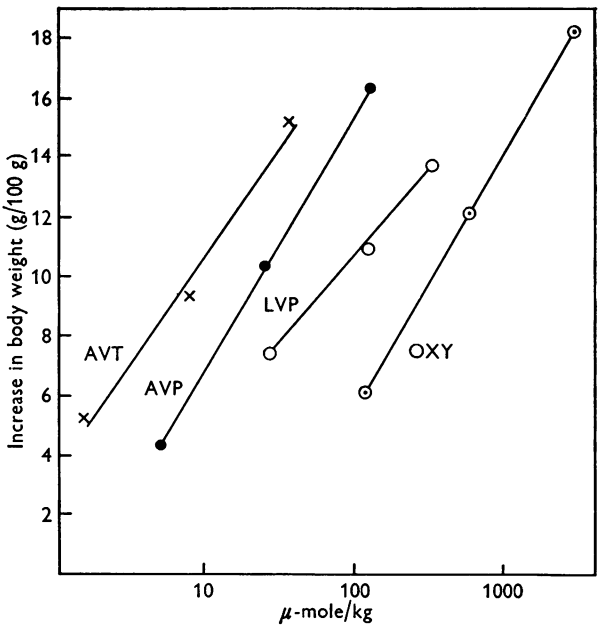

Fig. 3. Effect of neurohypophysial hormones on water retention by *Triturus alpestris*. Log-dose effect relationships. AVT, arginine vasotocin; AVP, arginine vasopressin; LVP, lysine vasopressin; OXY, oxytocin. Each point represents the mean for five animals: For comments see p. 439.

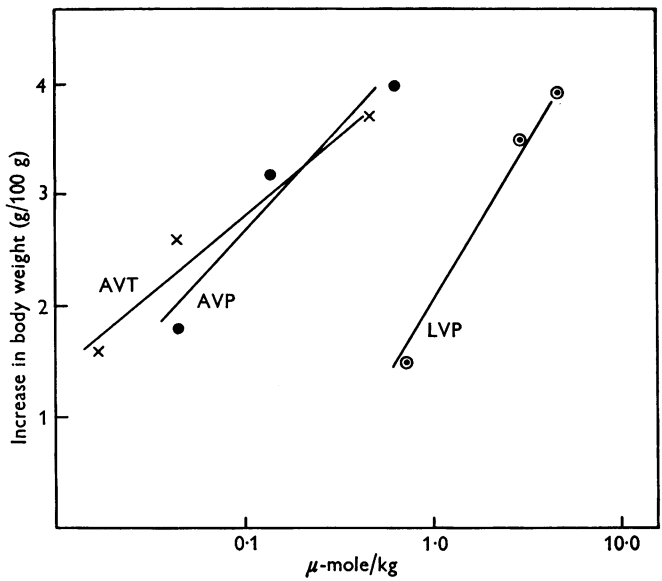

Fig. 4. Effects of neurohypophysial hormones on water retention by larval *Ambystoma*. Log dose–effect relation. AVT, arginine vasotocin; AVP, arginine vasopressin; LVP, lysine vasopressin. Each point represents the mean for five animals. For comments see p. 439.

arginine vasopressin that were about 100 times smaller than those needed in *Triturus alpestris*.

A small dose of arginine vasotocin (43 μ-mole/kg) in eleven specimens of *Necturus* produced a weight increase of $3{\cdot}6 \pm 0{\cdot}28$ g/100 g. An increase of this order of magnitude is apparently all this species if capable of since much the same weight of water ($3{\cdot}2 \pm 0{\cdot}47$ g/100 g) was also retained by ten animals after a much larger dose (129 μ-mole/kg) of vasotocin ($P > 0{\cdot}9$). As in the other urodeles species tested, oxytocin produced weaker responses than vasotocin: 110 μ-mole/kg raised the body weight of 10 mud puppies by $1{\cdot}7 \pm 0{\cdot}76$ g/100 g, a response which was smaller ($P < 0{\cdot}05$) than that given by 129 μ-mole vasotocin/kg. When the dose of oxytocin was lowered to 44 μ-mole/kg the mud puppies no longer responded (P for the difference between ten animals injected with oxytocin and ten animals injected with 0·6 % NaCl solution was $> 0{\cdot}9$) while, as shown above, water retention in mud puppies injected with the same dose of vasotocin was still maximal.

The site of action of neurohypophysial hormones in urodeles

Effects on the metabolism of water

I. *Triturus. Intact animals.* In a few preliminary experiments phenol red, a dye which is quickly excreted by frogs (Richards & Walker, 1930; Forster, 1940) was injected into *Triturus alpestris* in an attempt to demonstrate that vasotocin alters renal function. The first four intervals between the spontaneous voidance of coloured urine of eight newts injected with 7 μ-mole vasotocin/kg were timed, and the mean ($82 \pm 11{\cdot}4$ min) compared with the mean ($28 \pm 6{\cdot}6$ min) obtained in eight controls injected with the appropriate volume of 0·6 % NaCl solution. The difference is significant ($P < 0{\cdot}001$). On the assumption that the newts empty their bladders when approximately the same volume of urine has accumulated, these results suggested that the hormone decreased the rate of urine flow.

More precise information was obtained when the urine was collected in a rubber bag attached to the animal (see Methods). Tables 1 and 2 show that vasotocin (7 μ-mole/kg) decreased the urine volume markedly. Since the rate of water uptake remained much the same, the retention of water

TABLE 1. Effects of arginine vasotocin on the metabolism of water and sodium of *Triturus alpestris*. Means and S.E. for nineteen animals injected with 7 μ-moles arginine vasotocin/kg and twenty-three controls injected with appropriate volumes of 0·6 % NaCl solution

	Vasotocin	Controls	P
Water uptake (g/100 g/4 hr)	$18{\cdot}9 \pm 1{\cdot}98$	$26{\cdot}3 \pm 2{\cdot}57$	$< 0{\cdot}02$
Urine output (g/100 g/4 hr)	$10{\cdot}9 \pm 1{\cdot}66$	$26{\cdot}1 \pm 2{\cdot}7$	$< 0{\cdot}001$
Water retention (g/100 g/4 hr)	$8{\cdot}0 \pm 0{\cdot}91$	$0{\cdot}1 \pm 1{\cdot}05$	$< 0{\cdot}001$
Sodium output (μ-equiv/100 g/4 hr)	$42 \pm 15{\cdot}0$	$113 \pm 11{\cdot}3$	$< 0{\cdot}01$
Sodium concentration in urine (m-equiv/l.)	$4{\cdot}4 \pm 0{\cdot}82$	$4{\cdot}6 \pm 0{\cdot}52$	$> 0{\cdot}9$

was mainly produced by the renal action of the hormone. The same tables show also that vasotocin decreased the urinary sodium output but that the sodium concentration of the urines remained much the same.

Isolated skin. In agreement with the results *in vivo* high concentrations of vasotocin (29 μ-mole/l.) or arginine vasopressin (46 μ-mole/l.) failed to alter the rate of net water flux through pieces of isolated skin of either *Triturus alpestris* or *T. cristatus* (Table 3).

TABLE 2. Effects of arginine vasotocin on the metabolism of water and sodium of *Triturus cristatus*. Means and S.E. for eight animals injected with 7 μ-moles arginine vasotocin/kg and 12 controls injected with 0·6 % NaCl solution

	Vasotocin	Controls	P
Water uptake (g/100 g/4 hr)	19·6 ± 2·6	19·4 ± 2·0	> 0·9
Urine output (g/100 g/4 hr)	9·3 ± 1·6	19·2 ± 3·0	< 0·02
Water retention (g/100 g/4 hr)	10·6 ± 2·2	0·2 ± 0·75	< 0·001
Sodium output (μ-equiv/100 g/4 hr)	48 ± 13·2	106 ± 20·5	< 0·05
Sodium concentration in urine (m-equiv/l.)	5·6 ± 1·9	5·6 ± 1·1	—

TABLE 3. Rate of water transfer across isolated skin of urodeles at an osmotic gradient of 220 m-osmole/l. with and without neurohypophysial hormones present. *Triturus alpestris* and *T. cristatus:* 3 experiments each with arginine vasotocin (29 μ-mole/l.) and 3 with arginine vasopressin (46 μ-mole/l.). *Ambystoma:* 6 experiments with vasotocin (82 μ-mole/l.) and 3 experiments with vasopressin (46 μ-mole/l.). *Necturus:* 112 μ-mole vasotocin/l. Means and S.E. Number of experiments in brackets

	Water transfer (μl./cm^2 skin/hr)		
Species	Controls	Neurohypophysial hormones present	P
Triturus alpestris	7 ± 0·9 (6)	8 ± 1·8 (6)	> 0·7
T. cristatus	7 ± 1·4 (6)	7 ± 1·0 (6)	—
Ambystoma: larval	4 ± 0·9 (4)	7 ± 1·5 (4)	> 0·2
metamorphosed	2 ± 1·0 (5)	5 ± 1·9 (5)	> 0·1
Necturus	3 ± 0·9 (4)	4 ± 0·7 (4)	> 0·3

Isolated urinary bladder. The urinary bladder of *Triturus* is small, holding fluid equivalent to about 2 % of the body weight only. The isolated bladder can transfer water along an osmotic gradient like the skin. However, in four experiments neither vasotocin (29 μ-mole/l.) nor arginine vasopressin (46 μ-mole/l.), added to the fluid on the serosal side of the bladder, produced an unequivocal alteration in the rate of water movement.

II. *Necturus. Intact animals.* Table 4 shows the results of experiments in which the urine of *Necturus* was collected in rubber bags for 5 hr after the animals had been injected with either vasotocin (26 μ-mole/kg) or with 0·6 % NaCl solution. This period was chosen because previous experiments had shown (Fig. 1*b*) that the hormone was active for this length of time. A small but significant volume of water (less than 2 % of the body weight)

was retained under the influence of the hormone but neither a significant effect on water uptake nor on sodium output could be demonstrated.

Isolated skin. Arginine vasotocin at a concentration of 112 μ-mole/l. on the dermal side of bags of *Necturus* skin did not alter the rate of water transfer significantly (Table 3).

Isolated bladder. The urinary bladder of *Necturus* was found to hold fluid equivalent to about 5 % of the body weight. The rate of water transfer along the osmotic gradient in five experiments was $14 \pm 3{\cdot}0$ μl./hr/bladder. In another five experiments when arginine-vasotocin (112 μ-mole/l.) was added, water moved at the rate of $16 \pm 2{\cdot}9$ μl./hr/bladder, in other words at much the same rate.

TABLE 4. Effects of arginine vasotocin on the metabolism of water and sodium of *Necturus*. Mean and S.E. for ten animals injected with 24 μ-mole arginine vasotocin/kg and nine animals injected with appropriate volumes of 0·6 % NaCl solution. Five animals injected with vasotocin and three controls excreted volumes of urine too small for analysis

	Vasotocin	Controls	P
Water uptake (g/100 g/5 hr)	$6{\cdot}9 \pm 0{\cdot}90$	$4{\cdot}9 \pm 0{\cdot}29$	$> 0{\cdot}05$
Urine output (g/100 g/5 hr)	$5{\cdot}2 \pm 0{\cdot}85$	$4{\cdot}8 \pm 0{\cdot}47$	$> 0{\cdot}7$
Water retention (g/100 g/5 hr)	$1{\cdot}7 \pm 0{\cdot}30$	$0{\cdot}1 \pm 0{\cdot}45$	$< 0{\cdot}01$
Sodium output (μ-equiv/100 g/5 hr)	$167 \pm 35{\cdot}3$	$177 \pm 34{\cdot}7$	$> 0{\cdot}9$
Sodium concentration in urine (m-equiv/l.)	$31 \pm 7{\cdot}7$	$32 \pm 5{\cdot}0$	$> 0{\cdot}8$

III. *Ambystoma. Isolated skin.* Table 3 shows that, as in *Triturus* and *Necturus*, arginine vasotocin did not alter water transfer through the skin of larval or metamorphosed axolotls significantly.

Isolated bladder. The urinary bladder of *Ambystoma* holds fluid equivalent to about 20 % of the body weight. Water movement through this large organ could be easily measured but was not increased by 112 μ-mole arginine vasopressin/l. Bladders from both larval and metamorphosed animals were tested.

Effects on sodium metabolism

I. *Triturus. Intact animals.* Net sodium movements between the animal and the surrounding fluid were measured while the newts were immersed in tap water (sodium concentration: 0·38 m-mole/l.). The animals usually lost sodium to the surrounding fluid but an injection of arginine vasotocin (115 μ-mole/kg) abolished this loss and caused an active uptake of sodium (Fig. 5). Tables 1 and 2 show that, in addition to this increased uptake of sodium, the neurohypophysial hormones decreased the urinary sodium output.

It might be objected that urinary sodium excretion had decreased because, during the collecting period of four hours, the urine in the rubber bag was in contact with the skin through which some of the sodium had

been re-absorbed. However, when experiments were so arranged (see Methods) that contact between the urine passed and the skin of the animal's tail was avoided, an injection of vasotocin produced a similar decrease in urinary sodium excretions as in the experiments in which this precaution had not been taken. The mean urinary sodium loss in fourteen specimens of *Triturus alpestris* injected with vasotocin (7 μ-mole/kg) was

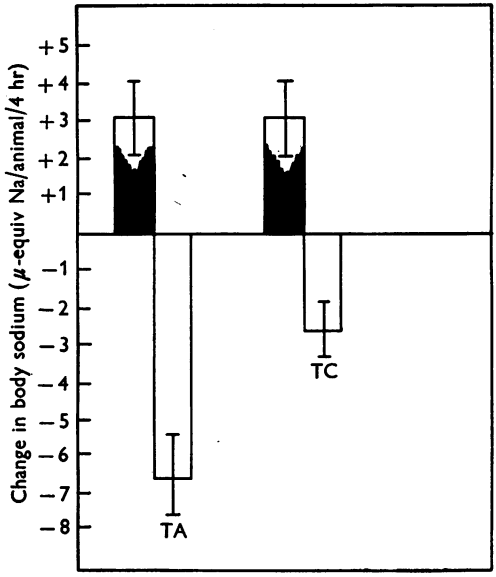

Fig. 5. Effect of arginine vasopressin on the sodium balance of *Triturus cristatus* (TC) and *Triturus alpestris* (TA). Ordinate: −sodium loss, + sodium gain. ■ animals injected with 115 μ-mole vasotocin/kg; □ control animals injected with 0·6 % NaCl solution. TC: means and S.E. (vertical bars) for fifteen newts in each group. TA: means and S.E. for twenty newts in each group. The animals were immersed in tap water (sodium concentration: 0·38 m-equiv/l.). Duration of experiment: 4 hr.

76 ± 18 μ-equiv/100 g/4 hr; control animals excreted 197 ± 41 μ-equiv/100 g/4 hr. The difference was again significant ($P < 0{\cdot}05$).

Isolated skin. In frogs and toads a potential difference can be measured between the dermal and epidermal surfaces of the skin. A similar p.d. was recorded across the isolated skin of *Triturus alpestris* ($22 \pm 5{\cdot}4$ mV (6 experiments)) and *T. cristatus* ($39 \pm 2{\cdot}8$ mV (6 experiments)). In anurans the SCC has been shown to be equivalent to the net sodium transport (Ussing & Zerahn, 1951) and to be increased by neurohypophysial hormones. Figure 6 shows that the SCC across *Triturus* skin is markedly influenced by the sodium concentration on the dermal side, suggesting that it is largely due to active transport of sodium. When the

sodium chloride in the fluid bathing the epidermal surface was replaced by choline chloride, the short circuit current decreased. Arginine vasopressin (46 μ-mole/l.) and arginine vasotocin (29 μ-mole/l.) (Fig. 7) rapidly increased the SCC across the isolated skin of both species of *Triturus*.

Isolated bladder. The urinary bladder of frogs and toads actively transports sodium in a similar manner as the skin (Leaf *et al.* 1958) giving rise

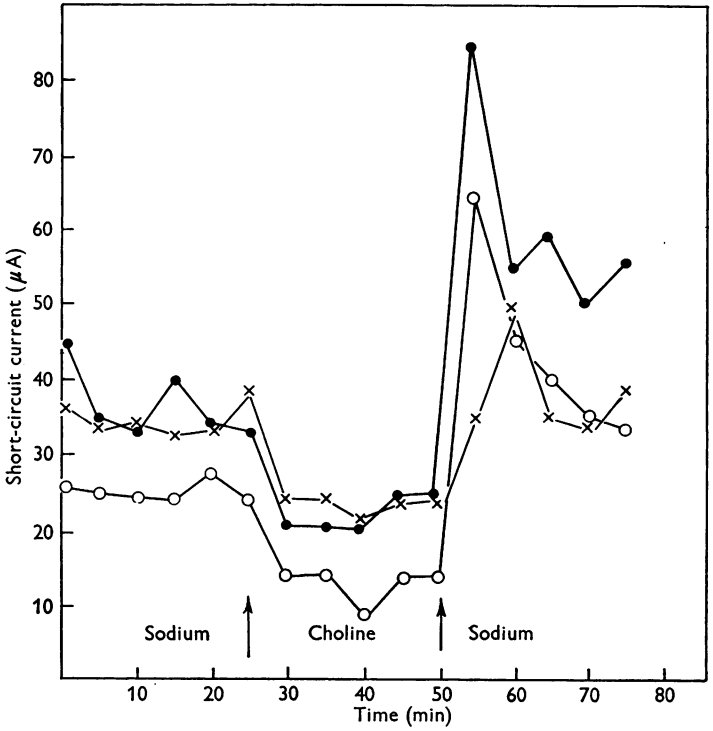

Fig. 6. Isolated skin of *Triturus alpestris*. Effect of replacing sodium on the epidermal side with choline (at arrow) on the short-circuit current across the skin; 25 min later sodium was restored. Skin samples from three different animals. For comment see p. 444.

to a p.d. However, no p.d. could be recorded across the isolated bladder of the newt (4 preparations). If, therefore, active transport of sodium is taking place, the resistance to the accompanying anions must be very different from that in the skin or anuran bladder.

II. *Necturus. Intact animals.* Net sodium loss from eleven animals kept in tap water was $10 \pm 3{\cdot}6$ μ-equiv/100 g/4 hr. Eleven mud puppies injected with a large dose of vasotocin (43 μ-mole/kg) lost $19 \pm 4{\cdot}2$ μ-equiv/100 g/4 hr. The difference is not significant ($P > 0{\cdot}05$).

Table 4 shows that the urinary sodium output in animals injected with a similar dose of vasotocin was unaffected ($P > 0{\cdot}7$).

Isolated skin. When undiluted Ringer's solution was placed on the epithelial side of *Necturus* skin, no p.d. could be recorded. A small p.d. ($12 \pm 1{\cdot}7$ mV) and SCC ($0{\cdot}2 \pm 0{\cdot}03$ μA/cm^2) was measured in five preparations bathed in Ringer's diluted 1:10. In view of these low values no attempt was made to investigate the effect of neurohypophysial hormones.

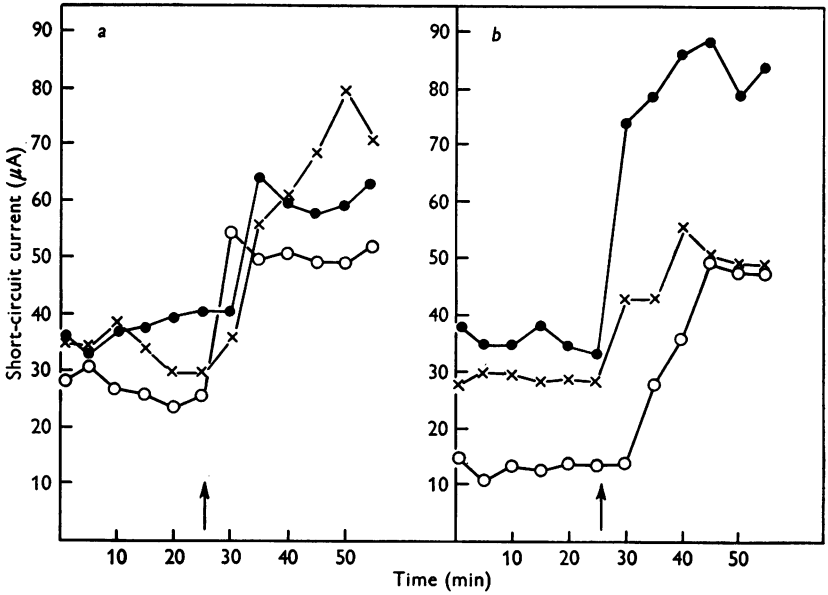

Fig. 7. Effect on short-circuit current across isolated newt skin of adding (at arrow) arginine vasotocin (30 μ-mole/l.) to the dermal side. *a*, *Triturus cristatus*; *b*, *T. alpestris*. Each curve was obtained for a skin sample from a different animal.

Isolated bladder. In contrast to the results in the isolated skin, a larger p.d. (39 ± 17 mV) and SCC ($9 \pm 3{\cdot}4$ μA/organ) were measured in four urinary bladder preparations. Ringer's solution, diluted 1:10, was used on the mucosal surface. In three bladders vasotocin (194 μ-mole/l.) failed to alter the short-circuit current.

III. *Ambystoma. Isolated skin.* As in *Necturus* no p.d. could be recorded across the isolated skin of larval axolotls when the dermal surface was bathed with Ringer's solution. A small p.d. ($7 \pm 1{\cdot}5$ (4 experiments) mV) was measured when dilute (1:10) Ringer's was used. The mean p.d. across the skin of metamorphosed axolotls was $43 \pm 5{\cdot}5$ (4 experiments) mV.

The SCC in the larval animals was too small to be measured accurately ($< 0{\cdot}5$ μA/cm^2). It could be reliably recorded in metamorphosed

Ambystoma (in which it amounted to $2{\cdot}5 \pm 1{\cdot}2$ (4 experiments) μA) but did not alter in the presence of arginine vasopressin (46 and 138 μ-mole/l.).

Isolated bladder. In four bladders of larval axolotls potential differences of 10, 12, 35 and 91 mV were recorded. The SCC in the last of these preparations was 55 μA and was not influenced by arginine vasotocin (86 μ-mole/l.). The p.d. obtained in two bladders of metamorphosed axolotls was 24 and 13 mV. The SCC was unaffected by arginine vasopressin (46 μ-mole/l.) or arginine vasotocin (86 μ-mole/l.).

DISCUSSION

The results presented in this paper confirm older reports (see Introduction) that neurohypophysial hormones produce small weight increases in the urodeles *Ambystoma* and *Necturus* kept in water. They show also that in some terrestrial newts (*Triturus alpestris* and *cristatus*) the maximum weight of water retained may amount to over 20% of the animal's body weight. Increases of this magnitude compare well with the large 'water balance' effects of posterior pituitary extracts in some species of frogs and toads (Heller & Bentley, 1963) and suggest a similar relation of these hormonal effects to habitat in urodeles as in anurans. The greatest response to arginine vasotocin was obtained in *T. alpestris* and in *T. cristatus*, both species that spend most of the year on land (Smith, 1951). The wholly aquatic *Necturus*, the mud puppy, showed a very weak response which, moreover, could only be obtained with a dose about 70 times greater than the hormone content of its neurohypophysis. (An extract of fourteen *Necturus* pituitaries contained 16 m-u. pressor and 21 m-u. oxytocic activity/gland. On the assumption that the pressor activity was due to vasotocin, the hormone concentration would amount to $0{\cdot}6$ μ-mole/kg body weight.) It may therefore be doubted whether the effects in this urodele species are of physiological significance. The maximal effects of vasotocin in axolotls were greater than those in the mud puppies although never as great as in the two species of *Triturus*. The axolotls used were the neotenous larvae which live permanently in water; the water-retaining action of the neurohypophysial hormones in metamorphosed *Ambystoma* seems to be more pronounced (Schreiber & Schreiber, 1950; H. Heller, unpublished results). Since very small amounts of vasotocin were needed to produce water retention even in larval *Ambystoma* (a dose of the order of $0{\cdot}001$ μ-mole/animal was sufficient to produce a significant effect) this hormonal effect may be suspected to play a useful role after metamorphosis. That vasotocin produces water retention in the larval axolotl, i.e. in a gill-breathing aquatic form may, at first glance, appear contrary to the assumed relation between magnitude of neurohypophysial effects on water metabolism and habitat. However, similar observations have been

made in the tadpoles of anurans, for example in the toad *Bufo bufo* by Howes (1940) and the frog *Heleioporus eyrei* by Bentley (1959)—suggesting that if a pronounced response is observed in the adult, some effect is also seen in the larvae.

While an influence of the neurohypophysis on the water metabolism can thus be demonstrated in both of the major subgroups of the amphibia, the urodeles and the anurans, the similarity of the over-all effects produced is in some sense deceptive. In all species of frogs and toads so far investigated the increase in body weight produced by neurohypophysial hormone has been shown to be due to a combination of increased water uptake through the skin and a decrease of urine volume. So far as can be judged from results in four species of urodeles, this subclass of the Amphibia lacks the former mechanism, i.e. no effect of the neurohypophysial hormones on water movement through the skin could be demonstrated even in the presence of a marked action on the kidney. The results of the experiments with isolated pieces of skin support this conclusion.

Another target organ for neurohypophysial peptides in anurans is the urinary bladder. When frogs and toads leave the water and, owing to the peculiar structure of their skin, begin to lose body water rapidly, the increased release of vasotocin (Levinsky & Sawyer, 1953; Janczo, 1955) leads to enhanced absorption of stored water from the bladder (Sawyer & Schisgall, 1956), so that presumably an undue rise of plasma osmotic pressure is prevented. This aid to osmoregulation seems to be absent in urodeles: the rate of water movement through the isolated bladder was not increased by neurohypophysial hormones in either *Triturus* or *Ambystoma*. Bladders of both larval and metamorphosed axolotls were used.

It seems therefore that in the urodeles which were tested water retention was produced by a purely renal effect. Hence the term 'water balance' or 'Brunn' effect should probably be restricted to frogs and toads in which water retention results from the interplay of several effector organs of the neurohypophysis. It should not be applied indiscriminately to the whole class of the Amphibia.

The mechanism of the antidiuretic effect of neurohypophysial hormones in urodeles needs further investigation although the decrease in urine flow without a change in urinary sodium concentration suggests a glomerular action of the hormone.

The species of urodeles investigated vary greatly in size and hence in the relation of body weight to body surface. Thus a renal inhibitory effect of the same magnitude would result in a greater increase in body weight in a smaller than in a bigger species provided that the permeability of the skin is of the same order. For example: the specific surface (cm^2 skin/g body weight) of *Ambystoma* (mean body weight = 36 g) was about 3 and

that of *Triturus* (mean body weight = 8·5 g) was approximately 5. Water uptake per unit body weight in *Triturus* would therefore be 1·7 times greater than in *Ambystoma*. Since, however, maximum water retention in metamorphosed *Ambystoma* after the injection of neurohypophysial hormones is about 7 g/100 g (H. Heller, unpublished work) and in *Triturus* frequently amounted to more than 20 g/100 g (and the permeability of the skin in the two species is much the same before and after administration of the hormones), the difference in specific surface cannot account for this discrepancy, suggesting that the antidiuretic effect in *Triturus* is more pronounced.

Dose-effect curves for some of the naturally occurring neurohypophysial hormones in *Ambystoma* and *Triturus* show that these species discriminate between most but not all of these peptides. Arginine vasotocin and arginine vasopressin seem to have much the same effect in larval axolotls, but the former hormone was somewhat more potent in *Triturus*. Both species distinguished clearly between arginine and lysine vasopressin. Oxytocin gave a weaker response than any of the other principles. The response to oxytocin of *Necturus* was also weaker than that to vasotocin. It seems therefore that the response of the renal receptors in these urodeles is mainly affected by substitutions in the tripeptide side chain: substitution in the pentapeptide ring (as, for example, the change from isoleucine in vasotocin to phenylalanine in arginine vasopressin) altered the potency relatively little, but the replacement of arginine in position eight in either arginine vasotocin or arginine vasopressin by leucine or lysine led to a marked loss of activity. It is interesting that Dicker & Eggleton (1961) found a similar influence of the basicity of the amino acid in position 8 on the antidiuretic potency of vasopressin analogues in man.

Neurohypophysial hormones affect the sodium metabolism of anuran amphibia by increasing sodium transport across the skin (Ussing & Zerahn, 1951), urinary bladder (Leaf *et al.* 1958) and renal tubule (Jard & Morel, 1963). Krogh (1939) showed that larval *Ambystoma* can actively take up sodium chloride from dilute solutions and Jørgensen *et al.* (1946) found that the injection of neurohypophysial extracts initially increased sodium excretion in these animals and that this loss was followed by a prolonged increase in uptake. We could not demonstrate any effects of neurohypophysial hormones on the short circuit current across the isolated skin or bladder of adult *Ambystoma* or the bladder of larval animals, and this suggests that these hormones affect the sodium metabolism by an action on the kidney (or possibly also on the gills in the larval form). Similarly, in the aquatic *Necturus* no effect of neurohypophysial hormones on the SCC across the isolated bladder could be demonstrated. Indeed in this species large doses of vasotocin affected neither the net sodium balance

of the intact animal nor the urinary loss of this ion. In the terrestrial newt *Triturus*, however, injections of neurohypophysial hormones decreased net sodium loss from the animals and promoted active sodium uptake. This effect can be accounted for by decreased excretion of the ion in the urine and active uptake through the skin, two sites also affected in the Anura.

The sites of action of neurohypophysial hormones on sodium and water metabolism parallel one another in most anurans, and this suggests the attractive possibility that the effects are caused by an action on the same receptors (Leaf, 1961). The effects on sodium and water may, on the other hand, be independent. Bourguet & Maetz (1961), for instance, found that the actions of various analogues of oxytocin on water and sodium transport across frog skin was not always strictly parallel, and Maetz (1963) has shown that oxytocin stimulates sodium but not water transfer across the isolated skin of *Xenopus*. The results of our experiments on the isolated skin of *Triturus* also emphasize the possibility of different receptors for the effects of neurohypophysial hormones on sodium and water movement, and the results on intact animals are consistent with this assumption.

The electrical p.d. across the isolated skin of the aquatic species examined was much smaller than in the terrestrial forms, and this difference extended even to larval and metamorphosed axolotls. The reason for this difference is unknown, but certain possibilities are worth considering. First, the skin of the aquatic animals may be more sensitive to the isolation procedures than that of the terrestrial forms; the inability of pieces of larval *Ambystoma* and *Necturus* skin to withstand undiluted Ringer's solution on the epidermal side may be a reflexion of this difference. Secondly, the basal rate of active sodium transport across the skin of these animals may be lower than that in terrestrial forms, but this seems unlikely in species living in an environment conducive to sodium loss. Lastly, compared with the terrestrial animals, the skin of the aquatic species may have a decreased resistance to the passage of chloride ions. This would decrease the electrochemical gradient against which sodium ions are transported and presumably increase the animal's ability to transport this ion.

SUMMARY

1. The actions of neurohypophysial hormones were tested in urodele species with different habitats. Maximum weight gains in animals kept in water were very small in the wholly aquatic *Necturus*, they were somewhat larger in larval *Ambystoma* but reached over 20 g/100 g body weight in two species of newts (*Triturus*) which live mainly on land.

2. The retention of water by these urodele amphibians in response to

the hormones was shown to be exclusively due to a renal action, in contrast to frogs and toads in which similar effects of these neurohypophysial polypeptides involve also the skin and the urinary bladder. No action of the hormones on water movement through the skin of intact *Triturus* or *Necturus* could be demonstrated; results on the isolated skin and bladder of *Triturus*, *Ambystoma* and *Necturus* were also negative.

3. Arginine vasotocin and arginine vasopressin proved to be the most potent hormones in *Triturus* and *Ambystoma*, followed by lysine vasopressin and oxytocin.

4. The isolated skin of *Triturus alpestris* and *T. cristatus* was found to transport sodium actively from the epidermis to the dermis. As in anurans, the rate of transport was increased by neurohypophysial hormones. The injection of some of these hormones into intact newts produced a retention of sodium due to decreased renal loss and increased uptake through the skin.

5. In *Necturus* and larval *Ambystoma* the short circuit current across the isolated skin was too small ($< 0{\cdot}5\ \mu A/cm^2$) to allow tests with neurohypophysial hormones. The short circuit current across the skin of metamorphosed *Ambystoma* was $2{\cdot}5\ \mu A/cm^2$ but was not altered by vasotocin or vasopressin.

6. In contrast to frogs and toads, the short circuit current across the urinary bladders of larval and adult *Ambystoma* and of *Necturus* were not affected by vasotocin or vasopressin.

7. A comparison between the actions of neurohypophysial hormones on the water metabolism of urodele and anuran amphibians suggests a similar relation between habitat and magnitude of response but since the mechanism of these effects in urodeles differs substantially from that in anurans it is proposed to restrict the term 'water balance' or 'Brunn' effect to the anuran amphibians.

Our thanks are due to Dr B. K. Follett for estimating the hormone content of *Necturus* pituitaries and for purifying and standardizing some of the hormone preparations used. We are indebted to Messrs G. J. Lane and D. Leathers for their unfailing technical assistance.

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