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SEASONAL CHANGES IN THE PERMEABILITY OF THE ISOLATED VESICAL EPITHELIUM OF *TESTUDO HERMANNI HERMANNI* GMELIN

M. GILLES-BAILLIEN

Department of Biochemistry, University of Liège, Liège (Belgium)

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SUMMARY

1. The vesical epithelium was cleared of muscular and connective tissues by dissection in *Testudo hermanni hermanni* Gmelin.

2. The water permeability of the vesical epithelium was lower in torpid than in aroused tortoises.

3. Antidiuretic hormones induced an increase (100%) in the net water flux measured across the vesical epithelium of torpid tortoises, while no effect was recorded on the net water flux measured in aroused tortoises.

INTRODUCTION

The permeability characteristics to water as well as to inorganic ions have been extensively studied in the urinary bladder of amphibians[1], and the effects of antidiuretic hormones on these permeability characteristics have formed the subject of elaborate investigations[2]. The urinary bladder of chelonians has been shown to behave rather similarly to the amphibian urinary bladder. The main studies concern an aquatic species, (freshwater) *Pseudemys scripta*[3-5]; it has been shown that Na^+ is actively transported from the mucosal solution to the serosal side, and a permeability to water has been exhibited. In contrast, unlike the amphibians, no effect of pituitary hormones either on water permeability or on active Na^+ transport could be detected[3]. BENTLEY[6] has published a paper concerning the urinary bladder of the terrestrial tortoise, *Testudo graeca*, and he also comes to the conclusion that neurohypophysial hormones remain without any effect either on the Na^+ transport or on the water permeability. The analysis of the urine composition in the terrestrial tortoise (*Testudo hermanni hermanni* Gmelin) has allowed us to suggest that the bladder must play an essential part in the reabsorption of salts and urea[7]. This role could be modified in hibernating animals since the urine composition differs in torpid and aroused tortoises. As a problem of water economy occurs during hibernation, the water permeability of the bladder must then be studied.

Our purpose in this paper is to study the permeability characteristics to water in the urinary bladder as a function of the season and of hibernation phenomena.

These investigations are carried out on the terrestrial tortoise, *T. hermanni hermanni* Gmelin. A preparation *in vitro* of isolated vesical epithelium, cleared of connective tissue, smooth muscle fibres and serosa, is obtained by dissection.

METHODS

The tortoises (*T. hermanni hermanni* Gmelin) are pithed and the shell is sawn open. The bladder is cut at the sphincter, emptied of urine and placed in an usual saline solution described below. The two lobes of the bladder are dissected with microdissection forceps under binocular microscope so as to obtain the isolated vesical epithelium. The removal of the muscular fibers, connective tissue and serosa allows us to obtain a preparation of vesical epithelium easy to set between two plexiglass compartments in order to study the water permeability by a microvolumetric technique[8]. How tortoises are able to live and undergo hibernation in Belgium (although originating from Yugoslavia) is described elsewhere[7].

The basic saline solution used for dissection or as serosal saline in the measurement of net water flux has the following composition (mM): NaCl, 112; KCl, 1.9; $CaCl_2$, 0.45; $MgSO_4$, 1; phosphate buffer (pH 7.0) ($Na_2HPO_4 + KH_2PO_4$), 1.7.

The antidiuretic hormones used are L8-vasopressin and arginine-vasotocin, both synthetic neuropeptides, which were supplied by Sandoz (Bâle).

RESULTS

The net water flux induced by an osmotic gradient was studied across the isolated vesical epithelium in torpid and aroused tortoises at different months of the year. Fig. 1 clearly shows that the net water flux is weaker in the torpid tortoises than in the aroused ones. If one compares results obtained within the same month, *e.g.* in April, in tortoises already aroused and in tortoises still torpid, the net water flux is markedly different; the same phenomenon appears in November at the beginning of hibernation.

The effects of antidiuretic hormones on the net water flux across the vesical epithelium was then tested in torpid and aroused tortoises. Table I shows that either L8-vasopressin or arginine-vasotocin (which constitutes with oxytocin the antidiuretic

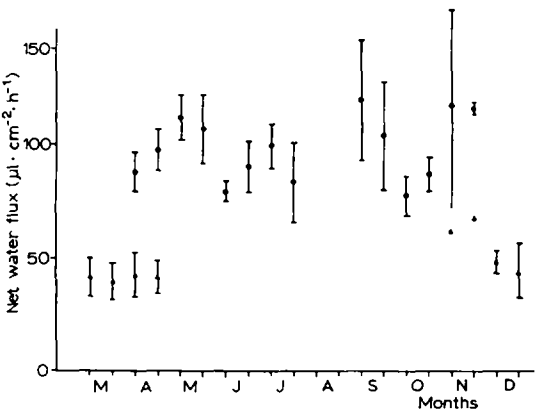

Fig. 1. Net water flux across the isolated vesical epithelium at the different months of the year. Conditions of experiments: serosal saline solution is the usual one; the mucosal saline solution is a 10-fold dilution of the serosal one. For each month two mean values have been established, each corresponding to the first or to the second experimental period of 0.5 h. ●, aroused tortoises; △, torpid tortoises.

principle of lower vertebrates) induces an increase of at least 100 % in the net water flux measured in torpid tortoises. No effect at all is recorded in aroused tortoises.

It must be noted that despite the considerable effect of L8-vasopressin or arginine-vasotocin on the net water flux in torpid tortoises, the values obtained never exceed those found in aroused tortoises, even the concentration of the hormone is increased up to 0.5 I.U./ml of saline.

TABLE I

EFFECT OF L8-VASOPRESSIN AND ARGININE-VASOTOCIN ON THE NET WATER FLUX ACROSS THE ISOLATED VESICAL EPITHELIUM

The net water flux measured in the presence of the usual osmotic gradient (*cf.* METHODS) is expressed in $\mu l \cdot cm^{-2} \cdot h^{-1}$ (mean values ± S.E.), n: number of experiments. For each experiment, two control periods of 0.5 h (C) and one experimental period of 0.5 h (E) during which L8-vasopressin or arginine-vasotocin is present in the serosal saline solution at a concn. of 0.1 I.U./ml. Serosal saline solution: the basic saline solution; mucosal saline solution: a 10-fold dilution of the serosal saline solution.

	Torpid			*Aroused*		
	C	*C*	*E*	*C*	*C*	*E*
L8-vasopressin	41.4 ± 8.6	39.6 ± 8.2 $n = 4$	86.2 ± 13.7	103.4 ± 6.1	104.7 ± 11.4 $n = 4$	105.4 ± 13.5
Arginine-vasotocin	42.6 ± 9.8	41.5 ± 7.2 $n = 3$	100.9 ± 22.5	83.5 ± 4.7	93.8 ± 5.9 $n = 4$	85.2 ± 12.5

TABLE II

EFFECT OF AN INCREASED NaCl CONCENTRATION ON THE NET WATER FLUX ACROSS THE ISOLATED VESICAL EPITHELIUM

The net water flux is expressed in $\mu l \cdot cm^{-2} \cdot h^{-1}$ ± S.E. Number of experiments in parentheses (n). Each experimental period lasts 30 min so that the longest experiment has been carried out for 6 h (12 experimental periods). Group A: A normal saline solution (112 mM NaCl) is added to the serosal compartment, while the same saline solution is diluted 10-fold in the mucosal saline solution. Group B: A saline solution with the NaCl concn. increased (200 mM) is added to the serosal compartment while a 10-fold dilution of the same saline solution is added in the mucosal compartment. Group C: The conditions of the experiments are the same as for Group B except that the fragments of vesical epithelium after dissection are preincubated for 5 h in a saline solution containing 200 mM NaCl, the osmotic gradient being applied after these 5 h.

	Experimental period No.					
	1	*2*	*3*	*4*	*5*	*6*
Group A	91.0 ± 15.2 (5)	97.1 ± 18.1 (3)	92.1 ± 10.6 (3)		64.5 ± 13.8 (3)	
Group B	170.9 ± 22.0 (7)	70.6 ± 11.9 (7)	82.5 ± 17.1 (7)	54.3 ± 12.8 (5)	48.9 ± 9.0 (4)	
Group C	87.4 ± 23.3 (3)	33.7 ± 2.2 (2)	20.3 ± 1.7 (2)	16.4 ± 1.2 (2)		

	Experimental period No.					
	7	*8*	*9*	*10*	*11*	*12*
Group A		70.5 ± 11.2 (3)		70.6 ± 15.1 (3)		64.9 (1)
Group B			27.5 ± 4.1 (3)	21.5 ± 4.0 (3)	23.7 ± 6.3 (2)	

Since an increase in Na^+ and Cl^- is recorded in the blood of hibernating tortoises[9], the effect of NaCl upon the net water flux was studied in the vesical epithelium of aroused tortoises. The first assays have shown a tremendous increase in the net water flux during the first experimental period (0.5 h) when the osmotic gradient is established as follows: serosal saline, the basic saline solution but where the NaCl concentration is 200 mM; mucosal saline, a 10-fold dilution of the serosal saline solution.

Experiments of long duration have shown that this increase was followed by a rapid decrease during the following periods. Table II shows the results obtained: Group A, results obtained with the usual osmotic gradient; Group B, results obtained when the osmotic gradient is established from a 200 mM NaCl saline solution; Group C, results obtained in the same experimental conditions as for Group B but after a previous incubation of the fragments of vesical epithelium in a 200 mM NaCl saline solution. The mean values obtained in each case have been plotted on a graph as a function of the number of the experimental period (Fig. 2).

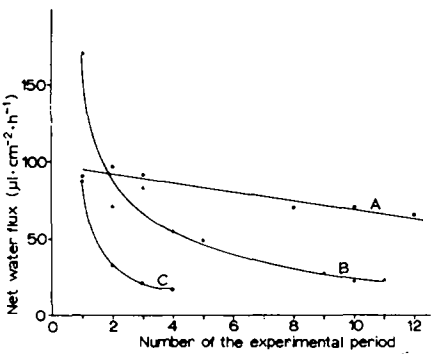

Fig. 2. Effect of an increased NaCl concn. on the net water flux across the isolated vesical epithelium. A, B and C have the same meaning as in Table II.

The following conclusions can be drawn: (a) In the presence of the usual gradient, the net water flux is represented by a straight line which, however, shows a progressive but rather slight decrease during time. (b) When the osmotic gradient is set up from a 200 mM NaCl saline solution a very high value of the net water flux during the first period is followed by an abrupt decrease during the ulterior periods, and after 5 h very low values are recorded. (c) A previous incubation in a 200 mM NaCl saline solution suppresses the initial increase in the net water flux, and a rapid fall immediately occurs.

Since the low values recorded after a certain time (in the presence of an osmotic gradient set up from a 200 mM NaCl saline solution), are of about the same magnitude as those recorded in hibernating animals, the effect of an addition of L8-vasopressin has been tested, but Table III shows that no effect is exerted.

Since urea concentration is also highly increased in the blood of hibernating tortoises[9], it was worth wondering if urea could also affect the net water flux. Table IV shows the results obtained when the net water flux is measured in the presence of an osmotic gradient set up from a saline solution enriched with 100 mM urea. The values obtained are quite similar to those found under normal conditions (Fig. 1 and

TABLE III

EFFECT OF L8-VASOPRESSIN ON THE NET WATER FLUX ACROSS THE ISOLATED VESICAL EPITHELIUM WHEN THE OSMOTIC GRADIENT IS ESTABLISHED FROM A 200 mM NaCl SALINE SOLUTION

The net water flux is expressed in $\mu l \cdot cm^{-2} \cdot h^{-1} \pm$ S.E. The control periods (C) 0.5 h each, are those measured 4 h after the beginning of the experiment when the net water flux has reached its lowest value. Three experimental periods (E) during which L8-vasopressin is present in the serosal saline solution at a concentration of 0.5 I.U./ml. Number of experiments is 5; serosal saline solution: basic saline solution but where NaCl is 200 mM; mucosal saline solution: a 10-fold dilution of the serosal saline solution.

C	*C*	*E*	*E*	*E*
23.9 ± 7.3	25.1 ± 2.5	25.9 ± 7.2	31.0 ± 7.5	26.5 ± 0.7

TABLE IV

NET WATER FLUX ACROSS THE ISOLATED VESICAL EPITHELIUM WHEN THE OSMOTIC GRADIENT IS ESTABLISHED FROM A SALINE SOLUTION CONTAINING 100 mM UREA

The net water flux is expressed in $\mu l \cdot cm^{-2} \cdot h^{-1} \pm$ S.E. For each experiment: three experimental periods of 0.5 h each; number of experiments is 10; conditions of experiment: the serosal saline solution is the basic saline solution but where urea is added at a concn. of 100 mM; the mucosal saline solution is a 10-fold dilution of the serosal saline solution. In the second group of experiments, fragments of vesical epithelium are preincubated for 5 h with the saline solution containing 100 mM urea (on both sides).

	Experimental period No.		
	1	*2*	*3*
Without preincubation	103.6 ± 17.6	113.8 ± 17.0	102.7 ± 8.3
With a preincubation of 5 h	158.6 ± 13.8	113.1 ± 24.4	114.9 ± 18.9

Table II); however, when the fragment of vesical epithelium is preincubated for 5 h in the saline solution containing 100 mM urea, the first experimental period gives a value which is in each case higher than those obtained during the following periods.

DISCUSSION

The hibernating tortoise stops eating, urinating and defecating. The bladder is however, still supplied with water through renal function since the volume of the bladder increases progressively during hibernation until it occupies nearly 50% of the shell cavity. Furthermore the fact that urea accumulates in the urine during hibernation[7] also indicates that excretion persists even though the metabolic activity is slowed down.

The bladder could therefore constitute a reserve of water during the whole hibernation, allowing the compensation of water loss through the respiratory tree, the water loss through the body surface being minimal in the case of chelonians. Several possibilities are offered to the animal in order to alleviate dehydration: (a) To increase the osmotic pressure of its blood and tissues, which is effectively the case[9–11]. (b) To recover water from the bladder. (c) To produce water metabolically.

The permeability characteristics of the bladder then had to be studied. Our

first aim was to obtain fragments of vesical epithelium cleared of connective and muscular tissues and of serosa, which constitute an obstacle to water or ion movement. Furthermore the problem was to study permeability of the vesical epithelium as exchanges between urine and blood and blood circulation comes in a close vicinity to the serosal border of the vesical epithelium through capillaries. In studies *in vitro*, investigations of the permeability of the bladder wall, underlying tissues included, are probably sullied by errors resulting from the presence of these underlying tissues, especially when contracted as is the case when the whole bladder is experimented on after having been emptied. This could perhaps explain the high difference recorded between the net water flux measured *in vitro* (9.3 $\mu l \cdot cm^{-2} \cdot h^{-1}$) and the net water flux measured *in vivo* (210 $\mu l \cdot cm^{-2} \cdot h^{-1}$) in the bladder of *Gopherus agassizii*[12].

The results we obtained for the net water flux across the isolated vesical epithelium of *T. hermanni hermanni* Gmelin (in aroused tortoises, about 100 $\mu l \cdot cm^{-2} \cdot h^{-1}$) are in fact much more similar to those measured in *G. agassizii in vivo*. However, one has to be careful in such comparisons, since an estimation of the surface of the vesical epithelium is very approximative. In the case of nonisolated epithelium, muscular contraction interferes and in isolated epithelium, the exceptional distensibility of this tissue can modify appreciably the real surface used for an experiment. This could explain, at least partially, the important standard error of the mean exhibited in our results, individual variations not being excluded.

When the net water flux is measured across the vesical epithelium of *T. hermanni hermanni* Gmelin at different months of the year, an abrupt increase is observed in April, and an abrupt decrease takes place in November, which coincides with the fact that tortoises awake from hibernation or become dormant (Fig. 1). The fact that the net water flux is very low in torpid tortoises is somehow in contradiction with what is expected, since water requirement seems to be more important in hibernating animals. However, this low net water flux can be controlled by antidiuretic hormones since L8-vasopressin (typical mammalian antidiuretic hormone) and arginine-vasotocin (which constitutes with oxytocin the antidiuretic principle of the Greek tortoise[13]), can enhance this net water flux by 100% (Table I). But it is of utmost importance to note that these two hormones have no effect on the net water flux in active tortoises and that the increased net water flux obtained with hormone in torpid tortoises never exceeds the basic values obtained in aroused tortoises. In active tortoises, we must therefore conclude that the hormone has no access to its action site in the vesical epithelium or that it has no more effect because the net water flux is at its maximal value. This result is, on the other hand, in agreement with the results of BENTLEY[6] who found no effect of pituitary hormones on the water permeability of the Greek tortoise bladder because he was probably working with active tortoises. In torpid tortoises, one has to assume first that a hibernating factor reduces the water permeability of the bladder mucosa and second that pituitary hormones become effective on this low water permeability.

Since all the measurements of the net water flux, either in torpid or in aroused tortoises, have been performed by establishing an osmotic gradient from a basic saline solution containing 112 mM NaCl, can it be said that the conclusions drawn above have a real biological significance?

The fact that Na^+ and Cl^- increase in the blood of hibernating tortoises[9] could induce variations in the net water flux. It has been shown for instance that the re-

placement of Na^+ by K^+ in the serosal saline solution induces a significant increase in the net water flux measured in the presence of an osmotic gradient across the urinary bladder of *Bufo marinus* L (ref. 14). On the other hand, in studying some properties of the isolated frog bladder in hyperosmotic solutions, BENTLEY[15] has shown that raising the concentration of NaCl by 100 mosM or by adding sucrose at a concentration of 100 mosM increases the water permeability, the osmotic gradient (directed the same as ours) being kept constant. However, a concentration of 100 mosM of urea has a much more mitigated effect.

Experiments carried out with aroused tortoises show that an increase in the NaCl concentration (200 mM) of the serosal saline solution induces a rapid increase immediately followed by a large decrease in the net water flux (Fig. 2), reaching values close to those obtained in torpid tortoises. An immediate decrease in the net water flux is observed when the fragment of epithelium is incubated beforehand in the saline solution containing 200 mM NaCl. When compared with the results of TERCAFS[14], we could presume that it is the Na^+ which is effective; however, further experimental support is needed.

These results are, in fact, in agreement with those of BENTLEY[15] since during the first experimental period (30 min) we measure an increase in the net water flux as he does and his results are based upon a measurement of the net water flux during one experimental period of 25 min. This first step perhaps reflects only the preliminary effort of the cells to reach a new steady state, however. The subsequent decrease in the net water flux could result, for instance, from a modification of the cationic content of the cells. However, the addition of antidiuretic hormones, after a low net water flux has been obtained in experiments performed with a basic saline solution containing 200 mM NaCl, remains without any effect. A high NaCl concentration in the serosal saline solution is thus insufficient to reproduce the events which take place in experiments performed with a basic saline solution containing 200 mM NaCl, and thus remains without any effect. A high NaCl concentration in the serosal saline is thus insufficient to reproduce the events which take place in experiments performed with vesical epithelium from torpid tortoises.

One important fact that still has to be borne in mind is that the increases in Na^+, Cl^- and urea concentrations of the blood appear progressively, already in September, in tortoises which are aroused. On the other hand, the change in the net water flux is abrupt and coincides with the beginning of the hibernation or the awakening of the tortoises. As to an increase in the osmotic pressure with urea, which increases tremendously in the blood of torpid tortoises, it appears to be without any significant effect on the net water flux, which is also in agreement with the results of BENTLEY[15] on frog bladder.

To conclude these experiments, the fact that NaCl is more concentrated in the blood of torpid tortoises is in agreement with a low water permeability in the bladder. However, this is not the governing factor which regulates this phenomenon, since this low permeability to water is demonstrated in the vesical epithelium of torpid tortoises where the experiment is performed with a saline solution containing 112 mM NaCl, which is the approximate concentration of NaCl in the blood of aroused tortoises. Therefore one has to postulate that the vesical epithelium of torpid tortoises acquires something different from that of aroused tortoises permitting the lowering of its permeability to water and the acquisition of a sensibility to pituitary hormones.

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