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Water Permeability and Lipid Composition of Toad Urinary Bladder: The Influence of Temperature

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Summary. The water diffusional permeability, its activation energy and the lipid composition were studied in urinary bladders from toads adapted to different temperatures. It was observed that the unidirectional water flux greatly depends on the temperature at which the experiments are performed. This dependence is greater in the animals adapted to higher temperatures. Toads adapted to cold show strong reduction in the activation energy for water diffusion permeability (from 11.4 ± 1.9 kcal·mol^{-1} to 4.4 ± 1.1 kcal·mol^{-1}) and an increase of 30% in the amount of total lipids from bladder epithelial cells. There were no significant changes in the phospholipid/cholesterol ratio, composition of the paraffinic chains or protein concentration between toads adapted to both temperatures. The possibility that water translocates through the mucosal border of the toad bladder by partitioning in the polar zone and diffusioning between the hydrocarbon chains of the membrane lipids and that cold adaptation would induce a stronger "packing" of lipids in the membrane is discussed.

In spite of the fact that the permeability of toad urinary bladder to water has been extensively studied, the molecular mechanism underlying water translocation is not yet completely understood. It is generally accepted that water movement across urinary bladder is regulated by a rate-limiting barrier at or near the mucosal border of the epithelium. On the basis of comparison between unidirectional and net water fluxes it has been proposed that water moves through aqueous channels across the epithelium and that antidiuretic hormone (ADH) increases the size or the number of such pores (Hays & Leaf, 1962*a*, *b*; Hays, Franki & Soberman, 1971). More recently, an alternative model in which water moves through the lipid barrier of the mucosal border by restricted diffusion has been considered (Hays & Franki, 1970; Parisi & Piccinni, 1973). The mucosal border has a lower permeability than the serosal

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membrane and different hypotheses have been evaluated to explain this difference. Among them has been considered the possibility that the lower permeability of the cell membrane forming the mucosal barrier is due to its lower cholesterol/phospholipid ratio (Cremaschi, Henin & Calvi, 1973). Then, variations in the permeability to water in different groups of animals could also be related to modifications in the type or in the amount of lipids of the luminal membrane.

Some relevant information on the type of interactions between water and the membrane during the translocation could be obtained by studying the activation energy of this process (Hays & Leaf, 1962*b*; Hays *et al.*, 1971; Eggena, 1972). In the toad urinary bladder, Hays *et al.* (1971) observed an activation energy above the one reported for water diffusion in bulk solution (4.6 kcal·mol^{-1}; Wang, Robinson & Edelman, 1953). This high activation energy has been alternatively attributed to the formation of icelike structures within the membrane or to significant interactions between water and the components of the hypothetical "water channel". Hays *et al.* (1971) also reported that the activation energy for water translocation remains constant over a large range of temperatures.

The present experiments were designed to correlate water diffusional permeability, its activation energy and the lipidic composition of toad urinary bladder epithelium. Animals adapted to different temperatures were employed on the knowledge that this adaptation strongly affects the response to ADH (Bourguet, 1967).

Materials and Methods

Toads (*Bufo arenarum, Hensel*) were obtained from a commercial supplier (Izaguirre, Buenos Aires, Argentina) and kept on damp earth. In each lot half of the animals were placed at an environmental temperature of 20 ± 1 °C, while the other half remained in a room at 4 ± 1 °C. After three weeks in those conditions toads belonging to both groups were pithed, their bladders removed, placed in saline (NaCl 112; KCl 5; Ca_2Cl 1; $NaHCO_3$ 2.5 mM in all cases, pH 8.1), and gassed with air. Each urinary bladder lobe was everted, attached to a polyethylene cannula and filled with about 6 ml of solution. The everted sac (mucosal side outwards) was immersed for 40 sec in a saline solution containing 10 μC of tritiated water. The bladders were then washed for 0.5 sec in a nonradioactive solution and transferred to a preweighed empty beaker where the sac was cut open and the serosal fluid collected. 500-μl samples of this fluid were analyzed in a liquid scintillation counter (Nuclear Chicago, model 8662). The volume of the sac was calculated from its weight, and the estimation of its surface area from this volume assuming a spherical shape. The unidirectional water flux was expressed in μl·cm^{-2}·hr^{-1}. The validity of the method employed has been discussed previously (Parisi & Piccinni, 1973). The temperature

of the bathing medium was adjusted with a Lauda K2RD model temperature-adjustable circulator and mixing was provided by magnetic stirring.

For the biochemical determinations, the bladders employed in the permeability measurements were pooled with others obtained from animals adapted during the same period to the same temperature. The bladder epithelial cells were isolated following the method of Jard and Bastide (1970): Frozen and thawed urinary bladders were cut into small pieces and incubated in buffer solution (pH 7.4) containing 100 mM Tris-HCl, 5 mM $CaCl_2$ and 40 μg/ml of collagenase (Sigma Chemical Co., A grade) during 30 min at 35 °C. The cells were separated by vigorous stirring and gauze filtration, and then washed and centrifuged to 2,500 rpm several times in saline solution (to avoid erythrocyte contamination). Proteins were measured according to Lowry, Rosebrough, Farr and Randall (1951). Total lipids were extracted (Folch, Lees & Sloane Stanley, 1957) and the lipidic phosphorus content of this extract was determined according to Chen, Toribara and Warner (1956). Cholesterol was measured by a modification of the method of Lieberman-Burchard (Stadtman, 1957). The paraffinic chains were studied by gas chromatography (Pye, England). All assays for biochemical determinations were performed in triplicate, and the results expressed in mg per urinary bladder for proteins and in μM/mg of cell protein for phospholipids and cholesterol.

Results

The Problem of Unstirred Layers in the Determination of Water Unidirectional Fluxes

Dainty and House (1966*a, b*) demonstrated that the unstirred layers present at the surfaces of epithelial barriers strongly modify water flux measurements. Studying this problem in the toad urinary bladder, Hays and Franki (1970) demonstrated that while in nonstimulated preparations the unidirectional water flux is not affected by stirring the bath, in ADH-stimulated bladders the determination of water fluxes strongly depends upon bath stirring. On these bases we decided to study the influence of bath stirring rate in our experimental conditions. The sac preparation was used with everted bladder sacs and the mixing of the mucosal solution was performed with a magnetic stirrer.

The serosal bath was not agitated and the actual rate of stirring was determined with a Baer JR3 model stroboscope.

Fig. 1 shows the effects of bath stirring on the unidirectional water flux on paired hemibladders, one of them under the action of antidiuretic hormone (Syntocinon Sandoz, 10 mU/ml). Even though only the mucosal bath was stirred in our experiments, these results confirm the observations of Hays and Franki (1970). Therefore, unstirred layers do not affect water unidirectional measurements in toad bladder in the absence of ADH. This is a consequence of the low permeability to water of the unstimulated mucosal border. Hays *et al.* (1971) reported that the activa-

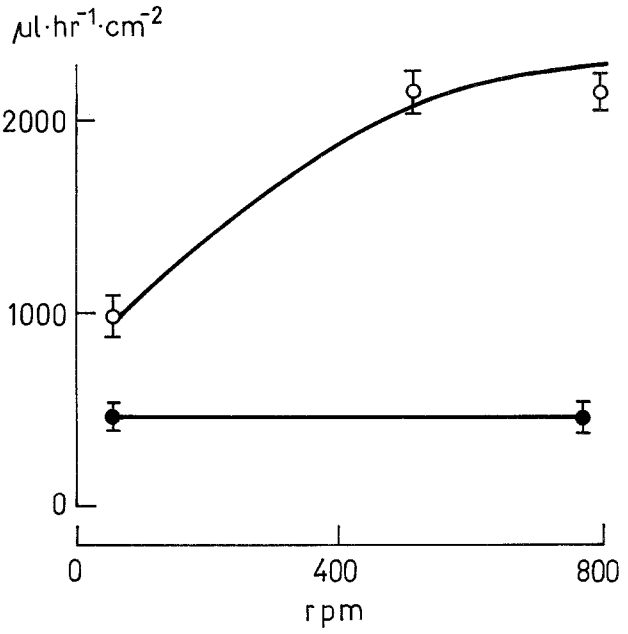

Fig. 1. Effect of stirring rate on measured water permeability. Open symbols are oxytocin-treated bladders; closed symbols are control bladders. Vertical bars are ± 1 SE

tion energy for water translocation is not affected by the presence of unstirred layers in the nonstimulated toad urinary bladder, a logical consequence of the independence of water fluxes upon bath stirring.

Results shown in Fig. 1 were similar for animals adapted either to 4 or 20 °C, in the case of unstimulated bladders. Since ADH was not used in the experiments described in this work, the bath was not vigorously stirred.

Effect of Temperature on the Unidirectional Fluxes of Water

Table 1 shows the experimental results obtained with bladders from animals adapted to 4 and 20 °C. The first set of experiments (type A) was performed at 6 and 22 °C. From these experiments it is clear that the unidirectional flux of water depends on temperature and that this dependence is greater for animals adapted to 20 °C. At 22 °C water permeability of animals adapted to cold is reduced as compared with the animals adapted to warm. The opposite is true for experiments performed at 6 °C.

Table 1. Unidirectional water fluxes across toad urinary bladder in toads adapted to different temperatures during a period of 21 days

Type	Exp. temperature	n	Water fluxes in $\mu l \cdot cm^{-2} \cdot hr^{-1}$		Mean diff.	p
			Animals adapted to			
			4 °C	20 °C		
A	6 °C		297±17	264±19	33	>0.1
	22 °C		501±21	628±44	−127	<0.01
B	6 °C		264±20	153±24	111	<0.01
	16 °C		326±23	293±18	33	>0.1
C	16 °C		262±15	271±22	−9	>0.1
	26 °C		373±17	443±25	−70	<0.05

In A, B and C the bladder water permeabilities of animals from the same batch adapted to 4 and 20 °C were compared. In toads adapted either to cold or warm water, permeability was measured at two experimental temperatures employing paired hemibladders in each experimental type.

According to Hays and Leaf (1962*b*) within the range of temperatures considered here the logarithm of the permeability coefficient is a linear function of the reciprocal of absolute temperature. Following this method an activation energy of 9.2 ± 1.4 kcal·mol^{-1} was obtained for animals adapted to 20 °C within the experimental range of 6 to 22 °C. This value is in accordance with the value of 8.4 ± 0.4 reported by Hays and Leaf (1962*b*) under similar conditions. However, when the activation energy for animals adapted to 4 °C was calculated it was found to be 5.3 ± 1.2 kcal·mol^{-1} (Table 2).

Table 2. Activation energy for water permeation in the toad urinary bladder of animals adapted to different temperatures during a period of 21 days

Range of temperatures	n	Animals adapted to		p
		4 °C	20 °C	
		(kcal·mol^{-1})		
6–22 °C	14	5.3±1.2	9.2±1.4	<0.05
6–16 °C	8	4.4±1.1	11.4±1.9	<0.01
16–26 °C	8	4.8±1.6	9.6±1.3	<0.05

To verify if the activation energy is constant over a wide range of temperatures, a second series of experiments was performed. The lot of animals adapted to 4 °C was divided in two groups. In each toad of the first group the permeability to water of one hemibladder was tested at 6 °C and the one of the other hemibladder at 16 °C. In the second group the experimental temperatures were 16 and 26 °C respectively. The same experimental design was employed with the lot of animals adapted to 20 °C. The results obtained are summarized in Table 1 (Types B and C) and in Table 2. It can be observed that at 6 °C the animals adapted to cold have a greater permeability than the animals maintained at a warmer environment while at 26 °C the opposite is observed. The mean activation energy calculated between 6 and 16 °C is not different from that calculated between 16 and 26 °C, in the animals previously adapted to 20 °C (11.4 ± 1.9 kcal·mol^{-1} and 9.6 ± 1.3 kcal·mol^{-1}, respectively). These values are also similar to those obtained in the first experimental set between 6 and 22 °C (9.2 ± 1.4 kcal·mol^{-1}).

When working with bladders from animals adapted to cold, the same strong reduction in the activation energy seen in the first experimental series was observed (Table 2). The values of 4.4 ± 1.1 kcal·mol^{-1} calculated between 6 and 16 °C, 4.8 ± 1.6 kcal·mol^{-1} calculated between 16 and 26 °C, and the value of 5.3 ± 1.2 kcal·mol^{-1} determined in the first set, between 6 and 22 °C, are not significantly different.

Changes in the Lipidic Composition Induced by Cold Adaptation

All biochemical determinations were performed on the isolated epithelial cells. No statistically significant differences between the total cell proteins from animals adapted to 4 °C and those adapted to 20 °C were observed (0.301 ± 0.024 *vs.* 0.338 ± 0.028 mg of protein per urinary bladder, respectively). Furthermore, no significant changes in the total mass of bladder tissue during cold adaptation was observed. The mean wet weights per urinary bladder were 419 ± 20 and 411 ± 25 for the animals kept at room temperature and for the cold adapted ones, respectively.)

A net increase in the amount of phospholipids per mg of cell protein was observed for animals adapted to cold as compared with animals adapted to a higher temperature (Table 3). The cholesterol concentration also seems higher for animals adapted to cold, but the experimental fluctuations were greater in this case. In general, large fluctuations were observed between different lots for animals adapted to the same temperature, both for phospholipids and cholesterol. This makes it very difficult

Table 3. Lipid composition in epithelial bladder cells from animals adapted to different temperatures during 21 days[a]

Exp.	Animals adapted to 4 °C				Animals adapted to 20 °C				A/B
	n	Phospholipid (1)	Cholest. (2)	1+2 (A)	*n*	Phospholipid (3)	Cholest. (4)	3+4 (B)	
1	12	1.25	0.61	1.86	12	0.71	0.39	1.10	1.69
2	19	0.95	0.95	1.90	19	0.72	0.79	1.51	1.26
3	28	1.16	0.63	1.79	28	0.69	0.47	1.16	1.55
4	23	1.31	0.63	1.94	25	0.74	0.81	1.55	1.25
5	18	0.94	0.70	1.64	19	0.82	0.52	1.34	1.22
6	30	0.96	0.38	1.34	32	0.73	0.30	1.03	1.30
7	17	0.51	0.45	0.96	17	0.50	0.42	0.92	1.04
8	24	0.47	0.25	0.72	23	0.42	0.18	0.60	1.20
$\bar{X}$		0.94	0.58	1.52		0.67	0.48	1.15	1.31
SEM		0.11	0.07	0.10		0.04	0.07	0.11	0.06
					Testing	3–1	4–2	B–A	↓
					p	<0.05	>0.1	<0.05	<0.0001

[a] Concentrations are expressed in μM per mg of epithelial cell proteins.

to establish variation patterns in the phospholipid/cholesterol ratio. In any case, after the analysis of 171 animals adapted to 4 °C and 175 adapted to 20 °C it can be concluded that there was a net increase in the amount of the total lipids from epithelial bladder cells with no significant change in the phospholipid/cholesterol ratio. (The expression "total lipids" here means phospholipids plus cholesterol, considering that the amount of other lipids in the membrane is negligible.)

In one set of bladders the analysis of the paraffinic chains was performed. Lipids from animals adapted to 4 °C were compared with those obtained from animals adapted to 20 °C (20 bladders in each case). No statistical significant differences were observed between both samples, and the saturation distribution was similar to that observed in other cases (egg yolk lecithin, for example).

Discussion

Recently, it has been proposed that water might traverse the luminal border of toad urinary bladder by restricted diffusion through the lipidic matrix (Hays & Franki, 1970; Parisi & Piccinni, 1973). However, there is presently no available direct information on the molecular bases of water translocation or its permeability increment induced by ADH. The

problem arises from the lack of knowledge of the considered barrier structure. Permeability of artificial undoped lipid bilayers is similar to that of cell membranes (Finkelstein & Cass, 1967). This supports the idea of similar translocation mechanisms in both membranes. Furthermore, it is possible to modify the lipid composition of these model membranes and studies correlating water permeability and lipid structure have been performed (Graziani & Livne, 1972). The objectives of this paper were to study the influence of the adaptation to different temperatures on the water permeability in toad urinary bladder and the possible modifications in the lipid composition of this structure. The information available from model membranes can be employed to interpret these results.

When toads are adapted to low temperatures the water diffusional permeability of its urinary bladder modifies and the activation energy of this process decreases. The determination of this last parameter has been employed to study the type of interactions between water and the membrane during translocation. Hays *et al.* (1971) have measured the activation energy for isotopic water diffusion across the bladder epithelium in the presence and in the absence of vasopressin. Both values were essentially the same: 10.7 ± 1.2 kcal·mol^{-1} for nonstimulated epithelia and 10.6 ± 1.1 kcal·mol^{-1} under ADH. These activation energy values are close to the values obtained here with animals adapted to 20° C (about 10 kcal·mol^{-1}). These values are also similar to those obtained for water permeation through artificial lipid membranes (Hanai & Haydon, 1966; Henn & Thompson, 1969).

The fatty acid composition of the toad urinary bladder lipids reported in the present paper is similar to that determined by Graziani and Livne (1972) in egg yolk lecithin. According to these authors, artificial membranes employing this last phospholipid show an activation energy of about 12 kcal·mol^{-1}. This value is similar to the value predicted from the solubility-diffusion model for water translocation (Hanai & Haydon, 1966). All these results favor the idea of a similar mechanism for water translocation in pure lipid bilayers and in the mucosal border of toad urinary bladder.

A strong reduction in the activation energy for water translocation was observed in the animals adapted to cold. This indicates an important change in the type of molecular interactions in the process of water translocation in these animals. Eggena (1972) has observed that the activation energy for the osmotic flux across the toad urinary bladder in the presence of ADH is considerably lower (4.1 kcal·mol^{-1} between

9 and 20 °C) than the activation energy for diffusional fluxes. To understand this observation, two hypotheses were proposed: (1) Water could be highly organized in an icelike structure within water channels. (2) In consequence, water-membrane bonds would be weaker than water-water bonds (assuming a nonpolar surface for the channel wall). In a diffusion process the rupture of water-water bonds would be the principal event explaining a high activation energy. In contrast, during osmotic flow water could "slide", preserving its own structure through the channel. The reduced value of the activation energy of this process would represent ruptures between the water core and the nonpolar channel wall. In summary, according to Eggena (1972) when the participation of membrane-water interactions increase, the energy of activation would become smaller. On the contrary, an increase in the relevance of water-water interactions would lead to higher activation energies. On the grounds of these speculations membrane-water interactions would become more important than water-water interactions in cold-adapted animals, as compared with warm-adapted ones.

An increase in the total amount of lipids of the epithelium with no significant changes in the phospholipid/cholesterol ratio is also reported here. This increase in the cell lipids was not paralled by a change in the protein concentration of the bladder epithelial cells and preliminary electron-microscope studies did not show a large deposit of lipids in the "cold" bladders (C. Vasquez and M. Parisi, *unpublished results*). It is not easy to correlate the observed variations in the physiological determinations with these biochemical observations, especially because there is no experimental evidence on the location of the lipid concentration increase within the cell. One could assume that the lipid increase reflects an increase in the amount of membrane lipids in each cell. It could then be concluded that lipids must be more packed in the membrane, reducing the "mean cross-section" of the water pathways. This would increase the relevance of membrane-water interactions and, according to the previous stated speculations, would explain the reduction in the activation energy. However, a modification in the polar heads of the phospholipids during cold adaptation cannot be discarded and this would also affect the partition of water in the membrane (Graziani & Livne, 1972).

It can be concluded that cold adaptation strongly modifies the molecular interactions between water and toad urinary bladder during water translocation. Further experimental work is necessary to clarify the observed variation in lipid composition.

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