

Temperature Dependence of Vasopressin Action on the Toad Bladder

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ABSTRACT Toad bladders were challenged with vasopressin at one temperature, fixed on the mucosa with 1% glutaraldehyde, and then subjected to an osmotic gradient at another temperature. Thus, the temperature dependence of vasopressin action on membrane permeability was distinguished from the temperature dependence of osmotic water flux. As the temperature was raised from 20° to 38°C, there was a substantial increase in the velocity of vasopressin action, but osmotic flux was hardly affected. In this range of temperature the apparent energy of activation for net water movement across the bladder amounted to only 1.2 kcal/mole, a value well below the activation energy for bulk water viscosity. It is suggested that osmotic water flux takes place through narrow, nonpolar channels in the membrane. When the temperature was raised from 4° to 20°C, both vasopressin action as well as osmotic water flux were markedly enhanced. Activation energies for net water movement were now 8.5 kcal/mole (4°–9°C) and 4.1 kcal/mole (9°–20°C), indicating that the components of the aqueous channel undergo conformational changes as the temperature is lowered from 20°C. At 43°C bladder reactivity to vasopressin was lost, and irreversible changes in selective permeability were observed. The apparent energy of activation for net water movement across the denatured membrane was 6.6 kcal/mole. Approximately 1 μ smol of NaCl was exchanged for 1 μ l of H₂O across the denatured membrane.

INTRODUCTION

The antidiuretic hormone, vasopressin, is thought to promote water flux across the isolated toad urinary bladder by increasing the permeability to water of a rate-limiting barrier at or near the mucosal cell face of the bladder epithelium (10, 11, 13). This increase in membrane permeability to water has been attributed by Hays et al. (6) to an increase in the number of small aqueous channels in the membrane rather than to an increase in their size as had been postulated previously (7). These conclusions were drawn from the observation that the energy of activation for isotopic water diffusion across the bladder epithelium was essentially the same in the presence and in the absence of vasopressin. Both values were well above the 4.6 kcal/mole reported

by Wang et al. (16) for diffusion of water in bulk solution. Such high activation energies have been explained (4-7) as resulting from the extensive bonding of water with the components of the aqueous channel or from the formation of icelike structures by water within the membrane. It is not possible on the basis of the diffusion data alone to determine whether the movement of individual water molecules is primarily restrained by water-water or by water-membrane hydrogen bonding.

To gain further insight into the nature and the extent of hydrogen bonding of water within vasopressin-stimulated toad bladders, studies on the temperature dependence of osmotic flow across this membrane were initiated. It was found that the number of aqueous channels in the membrane fluctuated with changes in temperature when vasopressin was present in the medium. This phenomenon made it impossible to relate temperature-sensitive changes in net water flux to changes in the frictional resistance encountered by water within a given set of aqueous channels. In order to keep constant the number of aqueous channels in the membrane with changes in temperature, bladders were fixed on the mucosa with a 1% solution of glutaraldehyde after they had been exposed to vasopressin. The temperature dependence of net water movement across the fixed bladder was then taken as a measure of the frictional resistance which water encounters when passing through a given set of vasopressin-induced aqueous channels. This approximation seems justified in the temperature range 4°-38°C where very little water is found to flow across bladders that have been fixed in the absence of vasopressin. The frictional resistance encountered in the vasopressin-stimulated membrane was found to differ significantly from the frictional resistance which one would expect in large channels, where water retains the physical properties of ordinary bulk water. In physiological ranges of temperature, activation energies for osmotic flux of water across the bladder wall were well below the activation energy of 4.6 kcal/mole for bulk water viscosity (16); at temperatures below 10°C activation energies for osmotic flux exceeded that value. The observation that the energy of activation for osmotic flux is not constant throughout the range of temperatures studied has indicated that the morphology and/or polarity of aqueous channels changes with temperature. At room temperature the energy of activation for osmotic flux was found to be considerably lower than the activation energy for diffusional flux reported by Hays et al. (6), which has been interpreted to indicate that different types of bonds must be broken for these two types of fluxes to take place in the membrane. Thus diffusion may depend primarily upon rupturing water-water hydrogen bonds, whereas osmotic flux may depend primarily upon breaking water-membrane hydrogen bonds. In a nonpolar channel water-membrane interactions would presumably be weak, but water-water interactions would be strong in view of the observation (2) that water has a tendency to assume an

icelike structure in the vicinity of nonpolar groups. On the basis of these considerations it has been concluded that net water movement in the presence of vasopressin takes place through narrow, nonpolar channels in the bladder wall. Water is visualized as flowing through these low resistance pathways by a process unlike Poiseuillean flow in that water would be more structured than in bulk solution and the interface between water and the membrane would not be stationary.

MATERIALS AND METHODS

Female toads, *Bufo marinus*, were purchased between May and July from the National Reagents Inc., Bridgeport, Conn., and maintained on moist peat moss. The animals were double pithed and their urinary bladders were isolated and tied to the ends of glass stalks so that the mucosa formed the inside of a sac which would hold about 7 ml of fluid. Osmotic water flux across the isolated bladder was measured gravimetrically according to the general method of Bentley (1). In a number of experiments bladders were challenged with vasopressin on the serosa and then "fixed" in different stages of permeability to water by replacing the mucosal fluid with 7 ml of a 1% glutaraldehyde solution in 0.05 M cacodylate buffer. The fixative had an osmolality of 215 mosmol/liter and a pH of 7.2. Bladders were usually fixed at room temperature where the fixative was allowed to react with the bladder mucosa for a period of 5 min. In one experiment (Fig. 6), however, the duration of fixation was shortened to 2.5 min or 2.0 min and extended to 10 min when the temperature of the fixative was 38°, 43°, or 4°C, respectively. After fixation bladders were rinsed twice with Ringer fluid, and the exact volume which the bladder could accommodate was measured. This volume was used to calculate the bladder surface area on the assumption that it was contained in a spherical bladder. Bladders were then filled with 7 ml of a 30 mosmol/liter solution which had all the components of Ringer fluid except for NaCl. Four bladders at a time were suspended by their stalks in a 200 ml serosal bath of 235 mosmol/liter Ringer fluid which was vigorously stirred by an air jet. The temperature of the serosal and mucosal fluids was regulated to within $\pm 1^\circ\text{C}$ by keeping the solutions in a constant temperature bath. Glutaraldehyde-fixed bladders were weighed at 15-min intervals (except for hemibladders *b* and *c* in Fig. 1); nonfixed bladders were weighed at 2-min intervals (except in Fig. 3). Bladders were wiped on a glass dish and weighed on a microbalance in dry air at room temperature with a consistency of ± 5 mg. The evaporative weight loss from the bladder assembly during a 15 sec weighing amounted to about 2 mg at 20°C and 5 mg when the bladder was at 38°C. Bladders were found to lose roughly 1000 mg in 15 min when responding maximally to vasopressin. The osmolality of the mucosal fluid was measured for each bladder at the end of a weighing period. The net increase in mucosal fluid osmolality was taken as an index of the serosal-to-mucosal flux of sodium chloride after corrections had been made for the concentrating effect of net water movement in the mucosal-to-serosal direction. From the net water and salt fluxes a mean osmotic pressure gradient was calculated for each bladder for a specific period. The hydraulic conductivity and permeability to sodium

chloride of the fixed bladder wall were determined from the net fluxes, the mean osmotic gradient, and the bladder surface area.

The Ringer fluid employed in the present study had the following composition, in millimoles per liter: NaCl, 110; KCl, 3.5; CaCl₂, 1; MgCl₂, 1; dextrose, 5.5; Trizma (Sigma Chemical Co., St. Louis, Mo.), 10, pH 7.4, osmolality 235 mosmol/liter. Commercial Pitressin (Parke, Davis & Co., Detroit, Mich.) was used as a vasopressin source.

RESULTS

Effect of Mucosal Glutaraldehyde on the Permeability to Water of the Bladder

The experiment shown in Fig. 1 illustrates that the action of vasopressin on the permeability to water of the bladder wall can be preserved by exposing the bladder mucosa for 5 min to 1% glutaraldehyde in 0.05 M cacodylate buffer. Bladders *a* and *b* were challenged with vasopressin in the presence of an osmotic pressure gradient, and net water flux was measured at 2-min intervals. As osmotic flux approached maximal levels in both bladders,

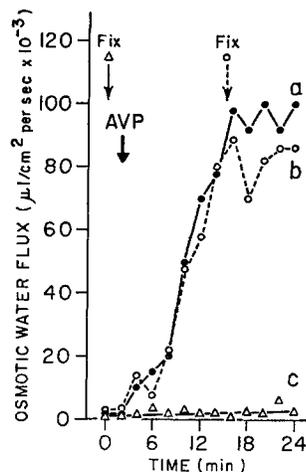


FIGURE 1

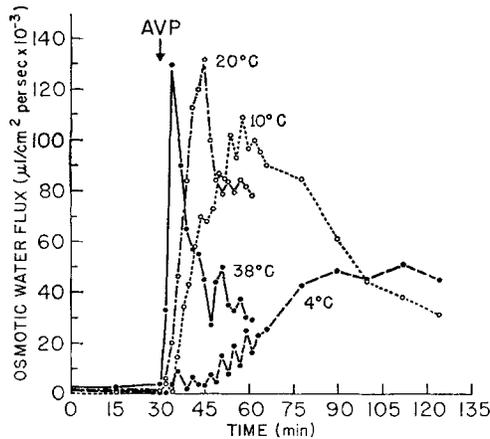


FIGURE 2

FIGURE 1. Effect of glutaraldehyde on water permeability of toad bladder. Three hemibladders were filled with 30 mosmol/liter of mucosal fluid each and placed into 235 mosmol/liter of serosal fluid containing 20 mU/ml of Pitressin (AVP). Hemibladder *a* served as the control; hemibladder *b* was exposed to 1% glutaraldehyde in 0.05 M cacodylate buffer on the mucosa for 5 min at $t = 12$ min and then placed in a hormone-free serosal bath; hemibladder *c* was fixed before being exposed to hormone.

FIGURE 2. Time-course of hydroosmotic response at different temperatures. Hemibladders were filled with dilute mucosal fluid, equilibrated for 30 min in Ringer fluid at 4°, 10°, 20°, or 38°C, and then challenged with 20 mU/ml of Pitressin (AVP). Each bladder was kept at the same temperature during the equilibration and challenge periods. Responses at 4°, 10°, and 38°C were normalized with respect to the 20°C response in contralateral hemibladders from the same toad.

bladder *b* was fixed and placed into a serosal bath which lacked vasopressin. Net water flux remained high in the fixed bladder, and this flux was now independent of the continued presence of hormone. Bladder *c* was fixed before hormone challenge. The fixed bladder did not respond to vasopressin. Furthermore, the fixation procedure did not increase the basal permeability to water of the bladder wall. These observations extend previous reports by Jard et al. (9) on glutaraldehyde fixation of frog bladders.

Temperature Dependence of the Hydroosmotic Response of the Bladder to Vasopressin

The time-course of the hydroosmotic response of the toad bladder to vasopressin was studied in the temperature range 4°–43°C. In Fig. 2 the responses at 4°, 10°, 20°, and 38°C are compared. Half-maximal responses to hormone were reached at 4°C after 35 min, at 10°C after 12 min, at 20°C after 6.5 min, and at 38°C after 2.3 min. Maximum levels of net water movement were similar at 38° and 20°C, but maximum flux was reduced by 20% at 10°C and by 63% at 4°C. The present observations at low temperatures are similar to previous findings by Rasmussen et al. (14) in the toad bladder.

When the temperature was raised from 38° to 43°C, there was a sudden loss in bladder reactivity to vasopressin (Fig. 3). At this temperature bladders remained relatively impermeable to water for approximately 45 min. Then both vasopressin-treated and untreated bladders became progressively more permeable to water, the vasopressin-treated bladders slightly more so. This heat-induced increase in water permeability was paralleled by an increase in salt permeability of the bladder wall. Approximately 1 μ osmol of NaCl penetrated the bladder wall from serosa to mucosa for every 1 μ l of H₂O moving in the opposite direction. The structural changes in the membrane matrix following prolonged exposure to 43°C temperatures were irreversible. Moreover, these bladders lost their capacity to respond to vasopressin when returned to a 20°C environment.

Temperature Dependence of the Velocity of Vasopressin Action

The experiment in Fig. 2 shows that the hydroosmotic response of the bladder to vasopressin develops more rapidly at 38° than at 20°C. This effect of the high temperature was in part due to the 30 min incubation period at 38°C before hormone challenge, as is illustrated by the experiment in Fig. 4. One bladder had been incubated at 38°C and subsequently challenged with vasopressin at 20°C. This bladder exhibited a more rapid and greater over-all hydroosmotic response than a paired bladder which had been incubated at 20°C and challenged at 38°C. The effects of a 4°, 20°, 38°, or 41°C incubation on bladder reactivity to a standard challenge with vasopressin are compared in Fig. 5. Bladder sensitization during a 30 min incubation period was optimal

at 38°C and minimal at 41°C. The observation that a 4°C incubation sensitized bladders relative to a 20°C incubation was unexpected.

The velocity of vasopressin action is influenced not only by the temperature of the prechallenge incubation bath, but also by the temperature of the challenge bath itself. This effect of temperature on the velocity of vasopressin

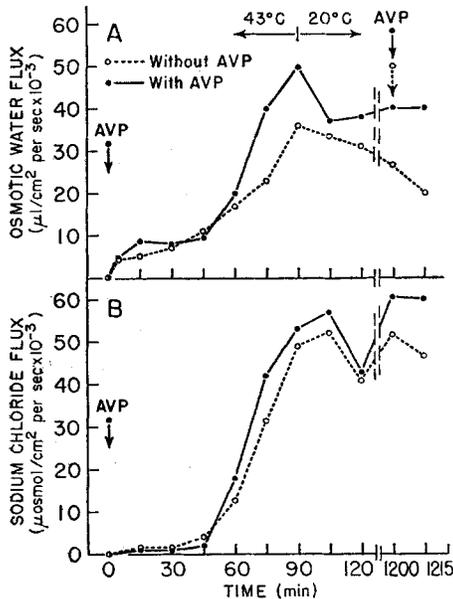


FIGURE 3

FIGURE 3. Effects of 43°C temperatures on bladder permeability to salt and water. Two sets of contralateral hemibladders were filled with dilute mucosal fluid and were placed in Ringer fluid at 43°C. One set (●—●) was challenged with 20 mU/ml of Pitressin (AVP); the other set (○—○) was not exposed to hormone. At $t = 90$ min both sets of bladders were placed in hormone-free Ringer fluid at 20°C and challenged at $t = 1200$ min with AVP. Values represent mean values from experiments on six toads.

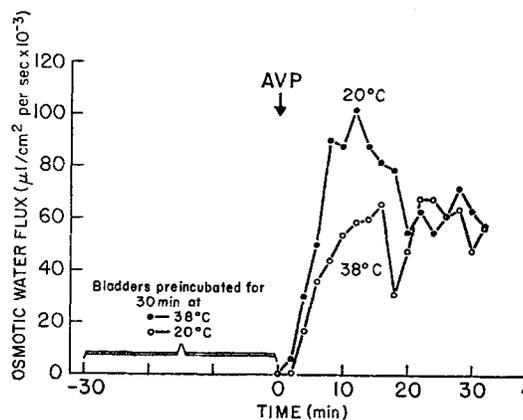


FIGURE 4

FIGURE 4. Bladder sensitization at 38°C. One hemibladder (●—●) was equilibrated for 30 min at 38°C and subsequently challenged with 20 mU/ml of Pitressin (AVP) at 20°C. The contralateral hemibladder (○—○) was equilibrated for 30 min at 20°C and then challenged with AVP at 38°C. The sudden drop in osmotic flux at $t = 16$ min at 38°C is due to manipulation of the bladder during the mucosal fluid change.

action was ascertained by measuring the hydraulic conductivity of the bladder wall after exactly 5 min of exposure to 20 mU/ml of Pitressin. To avoid interference by net water flux during the challenge period, bladders were filled with Ringer fluid which had the same osmolality as the serosal medium. To prevent temperature-sensitive changes during the fixation period the temperature of the fixative was adjusted to that of the hormone challenge bath. The data in Fig. 6 indicate that the velocity of vasopressin action increases pro-

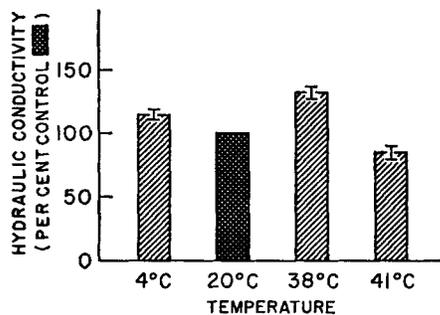


FIGURE 5

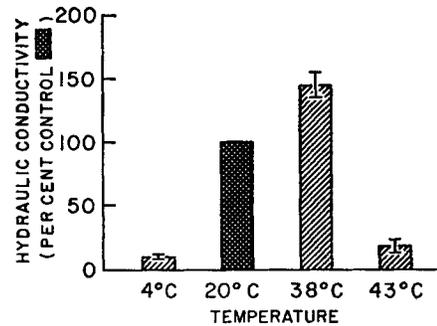


FIGURE 6

FIGURE 5. Effects of incubation on bladder reactivity. A control set of hemibladders was incubated in hormone-free Ringer fluid at 20°C for 30 min in the absence of an osmotic pressure gradient. Bladders were then exposed to 20 mU/ml of Pitressin for a period of 5 min at 20°C in the absence of an osmotic gradient. Bladders were fixed with glutaraldehyde for 5 min on the mucosa at 20°C. The hydraulic conductivity of the fixed bladder was measured and taken as 100%. Experimental sets of hemibladders were treated as their contralateral controls, the only exception being that the 30 min incubation period was carried out at 4°, 38°, or 41°C. Values are given as the mean and standard error of the mean from experiments on six toads at each temperature level.

FIGURE 6. Velocity of vasopressin action. A control set of hemibladders was incubated in hormone-free Ringer fluid at 20°C for 30 min and then challenged for 5 min with 20 mU/ml of Pitressin at 20°C in the absence of an osmotic pressure gradient. Bladders were fixed with glutaraldehyde for 5 min at 20°C on the mucosa. The hydraulic conductivity of the fixed bladders was then measured at 20°C and taken as 100%. Experimental sets of hemibladders were treated as their contralateral controls with the exception that the 5 min challenge period with AVP was carried out at 4°, 38°, or 43°C, and the hemibladders were fixed for 10 min at 4°C, for 2.5 min at 38°C, and for 2.0 min at 43°C. Values are given as the mean and the standard error of the mean from experiments on six toads at each temperature level.

gressively as the temperature is raised from 4° to 20° to 38°C. However, as the temperature is raised above 38° to 43°C, the velocity of hormone action diminishes abruptly.

Temperature Dependence of Osmotic Flux Across Glutaraldehyde-Fixed Bladders

Bladders were equilibrated for 30 min at 20°C, exposed for 5 min to vasopressin at 20°C, and fixed for 5 min with 20°C glutaraldehyde solution. The hydraulic conductivities of these bladders at 20°C ranged between 40 and 60% of the hydraulic conductivities of bladders exposed to saturating concentrations of vasopressin for an optimal period. Net water flux in response to an osmotic gradient was measured at 20°C in control bladders, and at 4°, 9°, 36°, or 38°C in matched contralateral bladders. The results in Fig. 7 show that the hydraulic conductivity of fixed bladders increased markedly as the temperature of the bathing fluids was raised from 4° to 9° to 20°C. However, a

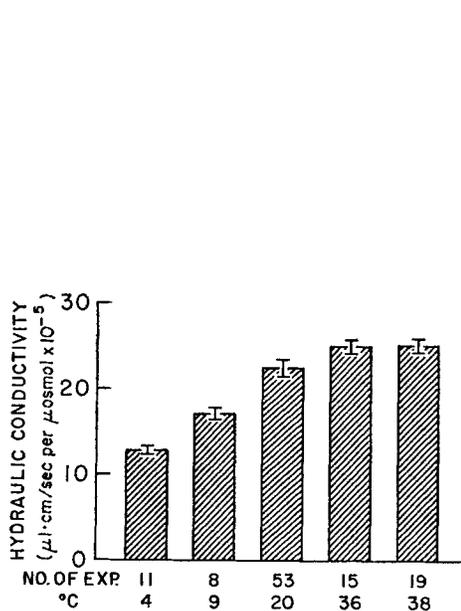


FIGURE 7

FIGURE 7. Temperature dependence of hydraulic conductivity of fixed bladders. Hemibladders were incubated for 30 min in hormone-free Ringer fluid at 20°C and then challenged with 20 mU/ml of Pitressin at 20°C for 5 min in the absence of an osmotic pressure gradient. Bladders were fixed on the mucosa with glutaraldehyde for 5 min at 20°C. The hydraulic conductivity of control hemibladders was measured during the first 15 min at 20°C. The hydraulic conductivity of experimental, contralateral hemibladders was measured during the same time interval at 4°, 9°, 36°, or 38°C. Values have been normalized with respect to controls and are expressed as mean hydraulic conductivities and the standard error of the mean from experiments on a minimum of eight toads at each temperature level.

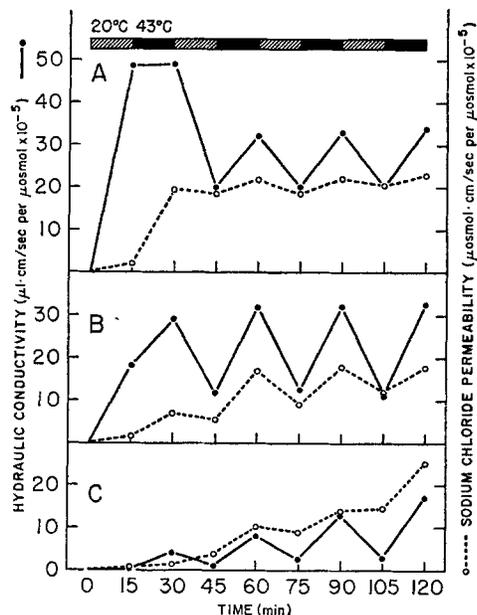


FIGURE 8

FIGURE 8. Denaturation of toad bladder permeability barrier. Hemibladders were challenged with 20 mU/ml of Pitressin in the absence of an osmotic gradient at 20°C for 12 min (A), 6 min (B), or 0 min (C). Bladders were fixed by exposing the mucosa for 5 min to 1% glutaraldehyde in 0.05 M cacodylate buffer. Mucosal-to-serosal osmotic water flux and serosal-to-mucosal sodium chloride flux were measured in 15-min cycles at 20°, 43°, 20°C, etc. The hydraulic conductivities and sodium chloride permeabilities of the fixed bladders were computed from net water and salt fluxes.

temperature increase from 20° to 36° to 38°C resulted in only a minimal increase in hydraulic conductivity. These values were employed for calculating the apparent energies of activation for osmotic flux in the usual manner (Table I). The apparent energy of activation for net water movement was 8.5 kcal/mole in the temperature range 4°–9°C, 4.1 kcal/mole in the range 9°–20°C, and 1.2 kcal/mole in the range 20°–38°C.

TABLE I
APPARENT ACTIVATION ENERGIES FOR OSMOTIC FLUX
OF WATER ACROSS GLUTARALDEHYDE-FIXED TOAD
BLADDERS

Hydraulic conductivity of fixed bladder at 20°C	Activation energies		
	4°-9°C	9°-20°C	20°-38°C
$22.5 \pm 1.2 \times 10^{-5}$ $\mu\text{l} \cdot \text{cm}/\text{sec per } \mu\text{osmol}$	8.5 ± 1.1 kcal/mole	4.1 ± 0.8 kcal/mole	1.2 ± 0.2 kcal/mole

The apparent activation energies for osmotic flux of water across the bladder wall have been computed from Arrhenius plots of the hydraulic conductivities shown in Fig. 7. Values given represent the mean and standard error of the mean of the slope when the logarithm of the hydraulic conductivity is plotted against the inverse of the absolute temperature. Bladders had been fixed on the mucosa with 1% glutaraldehyde in cacodylate buffer following exposure to 20 mU/ml of Pitressin at 20°C for 5 min.

TABLE II
HEAT-INDUCED CHANGES IN BLADDER PERMEABILITY TO SALT
AND WATER

Temperature	Period of exposure	Hydraulic conductivity	Activation energy for osmotic water flux	Permeability to NaCl	Salt/water exchange
°C	min	$\mu\text{l} \cdot \text{cm}/\text{sec per } \mu\text{osmol} \times 10^{-5}$	kcal/mole	$\mu\text{osmol} \cdot \text{cm}/\text{sec per } \mu\text{osmol} \times 10^{-5}$	$\mu\text{osmol}/\mu\text{l}$
20	0-15	19.5 ± 2.1	3.7	3.9 ± 1.7	0.20
43	15-30	31.2 ± 3.1	3.7	7.3 ± 2.2	0.23
20	30-45	17.2 ± 2.6	6.0	8.1 ± 0.8	0.47
43	45-60	36.5 ± 3.4	6.0	13.6 ± 1.3	0.37
20	60-75	13.9 ± 2.1	6.4	12.1 ± 1.3	0.87
43	75-90	31.2 ± 3.9	6.4	26.4 ± 3.0	0.85

Toad bladders were challenged with 20 mU/ml of Pitressin at 20°C for 5 min in the absence of an osmotic pressure gradient and then fixed at 20°C on the mucosa with glutaraldehyde. The hydraulic conductivities and permeabilities to sodium chloride of the fixed bladders were measured simultaneously at 15-min intervals at 20°, 43°, 20°C, etc. The apparent activation energies for osmotic flux of water across these bladders was computed from the slope of the logarithm of the hydraulic conductivity *versus* the inverse of the absolute temperature. Values are expressed as means and standard error of the mean from experiments in four hemibladders.

Denaturation of the Permeability Barrier of the Bladder

Bladders were exposed to vasopressin at 20°C for 0, 6, or 12 min and then fixed as detailed previously. This procedure yielded bladders with low, intermediate, and high permeability to water, respectively. The fixed bladders were subjected to marked fluctuations in temperature by exposing them for 15-min cycles to 20°, 43°, 20°C, etc. During these intervals net water and salt fluxes were measured (Fig. 8). Bladders which had been fixed at a near-

maximum response to vasopressin became less permeable to water and more permeable to salt. At equilibrium approximately $1 \mu\text{osmol}$ of NaCl penetrated the fixed bladder from serosa to mucosa for every $1 \mu\text{l}$ of H_2O that moved in the opposite direction. This equilibrium state was approached more gradually by bladders that had been fixed at intermediate or low stages of permeability to water. It is of interest that the selective permeability changes of the bladder fixed in the absence of vasopressin (Fig. 8 C) are similar to the changes observed in nonfixed bladders at 43°C (Fig. 3). In Table II, the progressive increase in the salt/water exchange ratio of fixed bladders exposed to cyclic temperature changes is compared with the progressive increase in the apparent energy of activation for osmotic flow of water across these bladders.

DISCUSSION

Glutaraldehyde is an effective cross-linking agent of proteins and polyhydroxy-alcohols first introduced by Sabatini et al. (15) into enzyme histochemistry as a fixative that preserves cellular fine structure without destroying the activities of a number of tissue enzymes. Both carbonyl groups of the dialdehyde can react rapidly with active hydrogens, amino, imino, and other reactive groups in protein, forming either intramolecular cyclic structures or intermolecular bridges when steric relations are favorable. The functional groups of proteins separated by distances greater than the pentamic chain of glutaraldehyde may be linked through methylene bridges formed by condensation of two or more aldehydes (3). The number and type of cross-links will determine the mechanical stability of the individual protein molecule and of the tissues in which proteins are enmeshed. In the present study it has been shown that the rate-limiting permeability barrier to water of the toad's urinary bladder can be "locked" in different stages of response to vasopressin by reacting the bladder mucosa for short periods of time with 1% glutaraldehyde. This observation suggests that the hormone triggers a structural rearrangement of the rate-limiting membrane which can then be stabilized by cross-linking membrane proteins and/or polyhydroxyalcohols. This suggestion does not imply that the actual sites which permit passage of water through the membrane, i.e. the so-called "aqueous channels," are made of proteins or polyhydroxy compounds. The confines of the aqueous channel could equally well consist of lipid material which has been stabilized by surrounding protein. Therefore, temperature-dependent changes in the geometry and chemistry of aqueous channels in the fixed membrane may be a consequence of temperature-dependent conformational changes in membrane proteins and/or of temperature-dependent phase transitions in membrane lipids.

Although vasopressin only increases the permeability to water of the toad bladder when applied to the serosal surface, several observations have indicated that the rate-limiting barrier affected by the hormone is located at or

near the mucosal surface (10, 11, 13). In order to fix the water permeability barrier of the bladder wall before inhibiting the action of hormone on this barrier, glutaraldehyde was applied to the mucosal bladder surface while keeping the serosal surface exposed to hormone. A 1% glutaraldehyde solution was usually allowed to react with the bladder wall for a period of 5 min at room temperature. These conditions were found to be adequate for fixing the hormone-sensitive permeability barrier without fixing the submucosal supporting tissues to an extent that the bladder wall becomes rigid. In one experiment (Fig. 6) the temperature of the fixative had to be adjusted to varied temperatures of the hormone challenge bath. To avoid fixing the entire bladder wall when the temperature of the fixative was 43°C, the duration of fixation was reduced from 5 to 2 min; to avoid inadequate fixation of the permeability barrier when the temperature of the fixative was 4°C, the duration of fixation was extended from 5 to 10 min. It must be admitted that these attempts to keep constant the degree of bladder fixation were not entirely justified because the same end results were obtained when bladders were challenged at 43° or 4°C and then fixed for 5 min at 20°C. Moreover, it is unlikely that the conditions of fixation employed in the present study are optimal for preserving the permeability properties of the bladder. Indeed it was found (Fig. 1) that net water flux across bladders fixed in the presence of vasopressin was diminished by 15–25%. Jard et al. (9) have reported a 50% reduction in osmotic water flow across frog bladders which had been fixed with 1% glutaraldehyde in 0.05 M cacodylate buffer following oxytocin stimulation. The difference in the preservation of hormone action on membrane permeability may be due to species differences or it may be due to the fact that these authors applied the fixative on the serosal bladder surface for a period of 15 min instead of applying the fixative on the mucosal bladder surface for only 5 min as was done in the present study. The observation (9) that glutaraldehyde-fixed bladders are slightly more leaky to sodium chloride than nonfixed bladders has been confirmed. Because of the tendency for this solute leak to dissipate the osmotic pressure gradient and thereby to diminish net mucosal-to-serosal water flux, it was necessary to measure water and solute fluxes simultaneously in the fixed bladder preparation and to correct for the influences of these two fluxes upon each other. Net water flux was measured with an accuracy of 0.5% provided that the bladder was maximally permeable to water and net water loss was measured over a 15 min interval. The precision of this method was, of course, proportionately less in experiments where bladders were not maximally permeable to water and where the bladder was weighed at 2-min intervals. Evaporative water loss from the bladder during weighing in dry air at room temperature amounted to 0.2% when the bladder was filled with fluid at 20°C and 0.5% when the bladder was filled with fluid at 38°C, given that the bladder was maximally permeable to water

and net water movement was measured for 15 min. Thus, errors due to weighing and due to evaporative water loss usually contribute little to the variability in net water flux encountered in different bladders or in the same bladder at different times.

The hydroosmotic action of vasopressin on the toad bladder is thought to involve a train of events initiated by activation of adenylyl cyclase at the receptor (12), mediated by an action of cyclic 3',5'-adenosine monophosphate (AMP) on protein kinase (8), and perhaps ultimately involving addition to the apical surface of material derived from secretion granules (11). The velocity of this sequence of reactions was found to be sensitive to the temperature of the bladder during vasopressin challenge (Fig. 6) as well as to the temperature of the bladder before challenge with hormone (Figs. 4 and 5). The net effect of both temperature-sensitive processes is to increase the permeability to water as the temperature is raised from 4° to 38°C, but to terminate abruptly the action of vasopressin on membrane permeability as the temperature exceeds 38°C and reaches 43°C. Since these effects of temperature on the membrane matrix are not related to the state of water within aqueous channels but rather to the relative number of aqueous channels in the membrane, measurements of activation energies for osmotic flux of water across the bladder wall in the presence of vasopressin are bound to be inaccurate. Thus, the activation energy of 4.6 kcal/mole reported by Hays and Leaf (7) for net water movement across the toad bladder in the presence of vasopressin in the temperature range 5°–31°C is probably an overestimate of the frictional resistance encountered by water in the membrane. Therefore, their conclusion that the core of water in aqueous channels of vasopressin-stimulated bladders possesses the physical properties of ordinary bulk water is uncertain.

In the present study the use of glutaraldehyde-fixed bladders has avoided the fluctuations in membrane permeability due to vasopressin, so that changes in net water movement with changes in temperature can be related to changes in the frictional resistance which water encounters in a stable population of aqueous channels. It must be pointed out, however, that the process of fixing the bladder may alter the geometry or chemistry of the aqueous channel in such a way that the frictional resistance encountered in these channels in the fixed bladder may not be identical to that encountered in the nonfixed bladder. Furthermore, it may be said that not all of the net water flux measured necessarily occurs through vasopressin-induced aqueous channels, but that a portion of the net flux occurs via hormone-independent pathways. While there is good evidence to suggest that "nonspecific" aqueous channels are opened up in fixed (Fig. 8 C) and nonfixed (Fig. 3) bladders after prolonged exposure to hormone-free Ringer fluid at 43°C, these non-specific channels probably play an insignificant role in bladders which are

exposed for short periods to temperatures below 38°C. The apparent energy of activation for osmotic flux of water across vasopressin-stimulated, glutaraldehyde-fixed bladders was found to be 1.2 kcal/mole in the temperature range 20°–38°C. Since this value is well below the activation energy of 4.6 kcal/mole for bulk water viscosity (16) the aqueous channels in the membrane must be sufficiently narrow to exert a substantial influence on the degree of hydrogen bonding of water, whether it is between neighboring water molecules or between water and free radicals in the membrane. Hydrogen bonds formed between water and the surfaces of the channel must be either weak or not very extensive if they alone are to account for the low frictional resistance that water meets in the channel. Alternatively, the hydrogen bond strength between water molecules could be less in water within narrow aqueous channels than it would be in bulk water, so that net water flux would be promoted by diminishing the shearing stress between moving layers of water. The weaker of the two types of hydrogen bonding within the channel will be the first to rupture and will, therefore, constitute the frictional resistance that is rate limiting for net water movement. It is not possible to decide on the basis of the osmotic flux data alone whether the water-water or the water-membrane bond is the weaker of the two bonds. Nevertheless, the latter possibility has been favored here as a working hypothesis for the present time. Accordingly, water could be highly organized in an icelike structure within the channel, and frictional resistance for net water movement would still be low if the structured core of water slides intact through aqueous channels in the membrane. Such channels would have to be essentially nonpolar to minimize hydrogen bonding at the water-membrane interface. In this context the suggestion by Frank (2) that nonpolar groups have a tendency to orient water into icelike structures is of interest, since it offers an explanation for the high energies of activation for diffusion of isotopic water across the bladder epithelium (6). Thus, the frictional resistance encountered in nonpolar aqueous channels in the vasopressin-stimulated toad bladder may be high for water diffusion and low for net movement of water, because the former process depends primarily upon breaking water-water hydrogen bonds, whereas the latter process depends primarily upon breaking water-membrane hydrogen bonds. These low resistance pathways induced by vasopressin in the membrane would be well suited for rapid transport of water from urine to interstitial fluid; at the same time, the icelike character of water during transport would hinder diffusion of solutes from interstitial fluid into the urine. Therefore, net movement of water in a relatively structured state would be more effective in stabilizing the osmotic pressure gradient across vasopressin-sensitive epithelia than water movement in bulk, according to Poiseuille's law.

One would expect changes in temperature to influence not only hydrogen bonding of water with water, or of water with the membrane, but to influence

as well the conformational state of the molecules which form the aqueous channel. Grigera and Cerejido (5) have shown a progressive increase in the activation energy for water diffusion across the outer barrier of the frog skin from 4.3 to 16.7 kcal/mole as the temperature is lowered from around 25° to around 0°C. Similarly, the apparent energy of activation for osmotic flux of water across the toad bladder was found to increase from 1.2 kcal/mole at 29°C to 8.5 kcal/mole at 6.5°C. Since the structure of bulk water changes little, if at all, between 4° and 38°C (16), the geometry and/or polarity of aqueous channels in the rate-limiting barrier of the toad bladder must not be constant within the range of temperatures studied. These conformational changes in the membrane are reversible except when brought about at high temperatures (Figs. 3 and 8). At 43°C the change is permanent, i.e., the membrane is denatured. In such denatured membranes osmotic flux of water takes on a different profile. Maximal rates of water flux per unit osmotic driving force are reduced by approximately 60%, and the apparent energy of activation for net water movement rises from 1.2 to 6.6 kcal/mole. Both observations point to an increase in the frictional resistance encountered by water in the denatured membrane. These changes in water permeability are associated with a marked increase in the serosal-to-mucosal movement of sodium chloride, so that for every 1 μ l of water that moves from mucosa to the serosa, approximately 1 μ osmol of sodium chloride moves in the opposite direction. This denatured state of the membrane is attained much more rapidly in bladders which have been exposed to vasopressin before fixation with glutaraldehyde than in bladders which have been fixed in the absence of hormone (Fig. 8). While this observation is consistent with the view that low resistance, selective aqueous channels are converted into high resistance, nonselective pathways upon heat treatment, it must be kept in mind that these nonselective pathways are formed also in the absence of preexisting aqueous channels. Although net water movement across the denatured bladder is less efficient than is net water movement across the intact bladder when vasopressin is present, a considerable amount of fluid is still reabsorbed from the urine across the denatured bladder.

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