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Selective Fixation with Glutaraldehyde of ADH-Induced Urea Permeability Sites in Toad Bladder (41639)

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Abstract. Toad bladders exposed to vasopressin (ADH) and then fixed on the mucosal surface with 1% glutaraldehyde were highly permeable to water and to urea compared to control bladders fixed in the absence of hormone. When identical conditions of fixation were used, but the concentration of glutaraldehyde was decreased to 0.25%, the ADH-induced increase in membrane permeability to urea was preserved whereas water permeability was not. About 74% of the hormone-induced urea permeability sites were preserved by glutaraldehyde and were stable to changes in temperature as suggested by a constant value for the activation energy of urea movement of 5.4 kcal/mole (4–33°C). In other studies bladders were exposed at low temperatures to 0.17% glutaraldehyde applied either to the serosal or the mucosal surface. The ADH-induced increase in membrane permeability to urea, bulk water, and tritiated water was well preserved with serosal fixation, but not with mucosal fixation. The observation that the urea pathway can be selectively preserved with 0.25% glutaraldehyde applied to the mucosa indicates that this structure is more accessible and (or) more sensitive to low-dose glutaraldehyde than is the ADH-induced water pathway. The observation that glutaraldehyde is more effective in stabilizing the ADH-induced urea channels from the serosal than from the mucosal surface indicates that these channels are not fixed at the extracellular surface of the apical plasma membrane. It appears, rather, that glutaraldehyde exerts its effects from an intracellular position, where it cross-links components of the urea channels at the cytoplasmic surface of the apical membrane and (or) inactivates the intracellular machinery responsible for the removal or dispersal of the ADH-induced urea permeability sites.

Vasopressin increases the permeability of the isolated urinary bladder of the toad to water (1) and to urea (2). The interaction of the hormone with receptors at the basolateral membrane stimulates the formation of intracellular cyclic AMP (3) which, in turn, triggers an increase in permeability of the apical membrane of the granular cells (4). The increased permeability to water of this membrane has been correlated with an increased frequency of intramembranous particle aggregates as seen by freeze-fracture electron microscopy (5, 6), although anatomical correlates of the urea permeability response to vasopressin have not been observed (7). There is mounting evidence which suggests that water and urea penetrate the rate-limiting barrier by separate pathways (8). Several investigations have shown, for instance, that urea movement can be selectively inhibited by agents such as cycloheximide (9), phloretin (10), thiourea (11), or amiloride (12). Other studies have shown that urea permeability can be selectively activated with low concentrations of vasopressin (13), and water permeability can, in turn, be increased without enhancing urea permeability with serosal hypertonicity (14) or with 8-bromoadenosine cyclic AMP (13).

The action of vasopressin to increase membrane permeability to water (15, 16) and urea (11, 17) can be largely preserved by fixing the bladder wall with glutaraldehyde. The present study has investigated the possibility of selectively fixing the vasopressin-sensitive urea permeability barrier by employing different concentrations of glutaraldehyde and applying the fixative to different sides of the bladder wall. The results to be discussed below indicate that the hormone-induced urea permeability sites are preserved at lower concentrations of glutaraldehyde than are the water permeability sites. Thus, when bladders were exposed to 1% glutaraldehyde on the mucosal surface both the urea and the water permeability response to vasopressin was stabilized for prolonged periods in the absence of hormone. However, when the concentration of

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glutaraldehyde was lowered to 0.25% (and keeping the duration and temperature of fixation constant) the water permeability response to vasopressin faded rapidly, whereas the urea permeability response was maintained. Other studies were carried out to learn if the increased sensitivity of the urea permeability sites to mucosally applied glutaraldehyde implies that this barrier is located superficially at the mucosal/urine interface. Therefore, the potency of glutaraldehyde as a preservative of hormonal action was compared with mucosal and serosal application under identical conditions. Bladders stimulated with vasopressin and fixed with 0.17% glutaraldehyde (for 10 min at 4°C) were considerably more permeable to urea when the fixative was applied to the serosal rather than the mucosal side. This observation suggests that glutaraldehyde fixes the hormone-induced urea channels not from the exterior (urinary) surface of the apical membrane, but rather from an intracellular locus which is reached more readily by diffusion across the basolateral rather than the apical plasma membrane. It is suggested that glutaraldehyde stabilizes the urea permeability sites in the apical membrane by cross-linkage of reactive groups at the cytoplasmic surface and (or) by interfering with the intracellular machinery responsible for the removal or dispersal of urea transport sites in the hormone target cell.

Materials and Methods. Experiments were carried out on female toads (*Bufo marinus*) from the Dominican Republic. Toads were doubly pithed and their urinary bladders isolated and tied to the ends of glass cannulae by the method of Bentley (1). Bladders were fixed with glutaraldehyde employing a modification of the technique used earlier (11) to stabilize the action of vasopressin on bladder permeability to urea. In the experiment depicted in Table I, bladders were challenged with vasopressin at a final concentration of 20 mU/ml at 21°C for 15 min with full strength Ringer's fluid bathing both bladder surfaces. The mucosal Ringer's solution was then replaced by Ringer's fluid at 21°C containing either 0.25 or 1.00% glutaraldehyde. The bladder mucosa was exposed to the fixative for 5 min, and during this time the bladder sac was immersed in Ringer's fluid at 4°C. This procedure minimizes the effects of the fixative on the contractile elements of the submucosa of the bladder wall. Following exposure to the fixative, the bladder was rinsed twice with Ringer's fluid and then filled with and suspended in McCoy's 5a Medium with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) adjusted to a tonicity of 235 mOsm/kg H_2O by addition of distilled water. This procedure was employed to removed unreacted glutaraldehyde from the bladder wall. Fixed bladders were incubated in hormone-free, modified McCoy's medium for 1.5 hr prior to use. The same general protocol was used for fixing bladders in the study depicted in Table II, with the following ex-

TABLE I. WATER AND UREA TRANSPORT ACROSS BLADDERS EXPOSED TO 1% OR 0.25% GLUTARALDEHYDE ON THE MUCOSAL SURFACE

Glutaraldehyde concentration	$P_f(H_2O) \times 10^{-4}$ cm × sec^{-1}		$P_d([^{14}C]urea) \times 10^{-7}$ cm × sec^{-1}		
	No ADH	With ADH	No ADH	With ADH	ADH-specific
1%	7.6 ± 2.2	109.6 ± 14.8	79 ± 52	234 ± 74	155 ± 54 (n = 5)
		$P < 0.001$			
0.25%	6.0 ± 2.2	18.9 ± 11.0	18 ± 5	148 ± 27	130 ± 29 (n = 6)

Note. Matched hemibladders were filled with Ringer's fluid and suspended in Ringer's fluid at 21°C for 15 min in the presence or absence of 20 mU/ml vasopressin (ADH). In the first series of experiments bladders were fixed with 1% glutaraldehyde, in the second series with 0.25% glutaraldehyde. The fixative was added to the mucosal Ringer's fluid at 21°C and the bladders suspended in Ringer's fluid at 4°C during a 5-min interval of fixation. Fixed bladders were thoroughly washed in modified McCoy's medium to remove glutaraldehyde and hormone. Both water- and urea-flux measurements were always carried out in the absence of hormone. The columns designated "No ADH" and "With ADH" refer to the absence or presence of vasopressin during the period prior and during fixation only. $P_f(H_2O)$ values were calculated from the osmotic water flow measured across fixed bladders filled with distilled water and suspended in a 233 m*M* mannitol/1 m*M* $CaCl_2$ solution at 21°C. Urea fluxes were measured from mucosa-to-serosa in the absence of an osmotic gradient.

TABLE II. WATER AND UREA TRANSPORT ACROSS ADH-TREATED BLADDERS EXPOSED TO LOW-DOSE GLUTARALDEHYDE ON THE MUCOSAL (HEMIBLADDERS A) OR SEROSAL (HEMIBLADDERS B) SURFACE

	$P_f(H_2O) \times 10^{-4}$ cm × sec^{-1}		$P_d([^{14}C]urea) \times 10^{-7}$ cm × sec^{-1}		$P_d(THO) \times 10^{-7}$ cm × sec^{-1}	
Toad No.	Hemibladder A	Hemibladder B	Hemibladder A	Hemibladder B	Hemibladder A	Hemibladder B
1	5.2	73.9	14	109	272	540
2	4.3	42.9	53	153	324	583
3	5.9	112.3	36	176	310	569
4	5.7	74.7	24	185	284	616
5	2.9	67.8	27	139	295	569
6	6.8	128.8	71	282	362	661
mean ± SD	5.1 ± 1.4	83.4 ± 31.5	38 ± 21	174 ± 59	308 ± 32	590 ± 43

Note. $P < 0.001$ between A and B hemibladders. Matched hemibladders A and B were filled with Ringer's fluid and suspended in Ringer's fluid at 21°C for 15 min in the presence of 20 mU/ml vasopressin (ADH). Hemibladders A were then exposed on the mucosal surface to 0.17% glutaraldehyde for 10 min at 4°C, while the B hemibladders were exposed on the serosal surface to the same concentration of glutaraldehyde also at 4°C for 10 min. Both hemibladders A and B were then equilibrated for 1.5 hr in hormone-free modified McCoy's medium and then suspended in a 233 m*M* mannitol/l m*M* $CaCl_2$ solution for water- and urea-flux studies. $P_f(H_2O)$ values were calculated from the osmotic water flow measured across bladders filled with distilled water. P_d values for urea and tritiated water were measured in the mucosal-to-serosal direction in the absence of osmotic water flow.

ceptions: (i) The concentration of glutaraldehyde was decreased to 0.17%. (ii) The temperature of the mucosal and serosal baths was kept at 4°C. (iii) The duration of fixation was extended to 10 min. (iv) The fixative was applied either to the serosal or the mucosal bathing solutions.

Isotopic fluxes were measured across the fixed bladders by filling each bladder with 7 ml of isotonic mannitol solution (233 m*M* mannitol/l m*M* $CaCl_2$) containing [^{14}C]urea or ^{3}H-water (THO) and suspending each bladder into a separate 25-ml serosal bath containing isotonic mannitol solution. Osmotic water flow across fixed bladders was measured by filling them with 7 ml of distilled water and suspending each into a separate 25-ml bath of isotonic mannitol solution. The osmotic water flux across these bladders was measured gravimetrically with a semimicro balance (Mettler, H43). At the completion of the experiment, the surface area of these bladders was estimated by determining the precise volume each hemibladder would accomodate at a hydrostatic pressure of 6 cm H_2O. This procedure provides a reasonable estimate of the optimal bladder capacity (18). From the bladder volume the surface area of the bladder wall was calculated on the assumption that the filled bladder conforms to a perfect sphere. In these experiments the bladder surface areas varied between 17.4 and 22.97 cm^2. Osmotic water flow across the bladder wall was expressed as a P_f value (i.e., $P_f(H_2O)$) in units of centimeters per second according to equations derived by Robbins and Mauro (19) and used by Schafer and Andreoli (20) in water flow measurements across isolated renal tubules. Implicit in these calculations is the assumption that the toad bladder wall has a reflection coefficient for mannitol of 1.0.

The Ringer's fluid employed in this study had the following composition, in millimoles per liter: NaCl, 110; KCl, 3.5; $CaCl_2$, 1; $MgCl_2$, 1; dextrose, 5.5; Trizma (Sigma Chemical Co., St. Louis, Mo.), 10; pH, 7.4. The osmolality of this solution was 236 mOsm/kg H_2O as measured with a vapor-pressure osmometer (Wescor). The glutaraldehyde employed (Sigma Chemical Co.) was taken from a fresh vial for each experiment, in order to avoid possible variations in the degree of fixation due to diminished potency of glutaraldehyde, which is known to occur with storage. Pitressin (Parke-Davis, Detroit, Mich.) was used as a source of synthetic 8-arginine-vasopressin.

Results. *Selective fixation of the urea permeability barrier.* Matched hemibladders were incubated at 21°C for 15 min in the presence or absence of vasopressin (20 mU/ml) and

fixed on the mucosa for 5 min with either 0.25 or 1.0% glutaraldehyde, as detailed under Methods. [^{14}C]urea fluxes were measured in the mucosal-to-serosal direction across these fixed bladders in the absence of vasopressin. As is shown in Table I, bladders which had been fixed with 1% glutaraldehyde were more permeable to urea than were bladders fixed with only 0.25% glutaraldehyde. This was true for bladders fixed in the presence or the absence of hormone. However, when the basal permeability to urea was subtracted from the permeability measured across bladders fixed in the presence of vasopressin, urea fluxes were similar in bladders fixed with different concentrations of glutaraldehyde. The urea flux due to hormone is designated as the ADH-specific P_d([^{14}C]urea) value in the table. Bladders fixed with 0.25% glutaraldehyde in the presence of vasopressin were about sevenfold more permeable to urea than controls fixed in the absence of hormone.

Osmotic water permeability $P_f(H_2O)$, of bladders fixed with 1 or 0.25% glutaraldehyde were similar for bladders fixed without hormone, but markedly different for bladders fixed in the presence of hormone. Thus, bladders fixed in the presence of vasopressin with 1% glutaraldehyde had a $P_f(H_2O)$ of 110 $\times 10^{-4}$ cm $\times$ sec^{-1}, whereas bladders fixed with only 0.25% glutaraldehyde had a $P_f(H_2O)$ of only 19 $\times 10^{-4}$ cm $\times$ sec^{-1}. Therefore, the vasopressin-induced increase in osmotic water permeability of the bladder wall is not preserved by mucosal fixation with 0.25% glutaraldehyde, whereas the hormone-induced increase in urea permeability is preserved in these same bladders.

Mucosal versus serosal fixation of the urea permeability barrier. Matched hemibladders were incubated at 21°C for 15 min in the presence of vasopressin (20 mU/ml) and then exposed for 10 min at 4°C with hormone to 0.17% glutaraldehyde on either the mucosal or the serosal side. Osmotic water fluxes, tritiated water fluxes, and [^{14}C]urea fluxes were then measured in the mucosal-to-serosal direction in the absence of hormone. As is shown in Table II, hemibladders A exposed to low-dose glutaraldehyde on the mucosal surface are significantly less permeable to water and urea than are the matched hemibladders B which had been exposed to glutaraldehyde under identical conditions on the serosal surface.

Stability of glutaraldehyde-fixed urea permeability sites. To evaluate the stability of glutaraldehyde-fixed bladders with regard to urea permeability, the experiments in Fig. 1 and Table III were carried out. In the experiment in Fig. 1 matched hemibladders had been fixed with 1% glutaraldehyde in the presence or absence of vasopressin as detailed in Table I. The hemibladder fixed in the presence of hormone and then removed to hormone-free media retained its high permeability to urea for about 20 hr. The bladder fixed in the absence of hormone remained relatively impermeable to urea for about 3 hr. However, permeability of this bladder increased about threefold following overnight incubation. When these hemibladders were subjected to an osmotic pressure gradient more than 20 hr after fixation, the bladder with a previous history of exposure to vasopressin was considerably more permeable to water than the control which had not been exposed to hormone before fixation. The experiment in Fig. 1 also illustrates the inhibitory effect of a low temperature on urea flux in the fixed preparation. Thus, both the basal and the ADH-stimulated urea flux was reduced to roughly one-half when the temperature was lower from 21 to 4°C.

In the experiment shown in Table III the effect of temperature on the rate of labeled-urea movement across bladders fixed with or without vasopressin with 0.25% glutaraldehyde has been studied. The temperature of the bathing solutions was progressively changed from 4 to 19 to 33°C and then back to 19°C. The apparent energies of activation were calculated for the ADH-specific urea fluxes and were found to be quite similar in these temperature ranges, suggesting that the urea permeability sites induced by ADH prior to fixation were stable with changes in temperature between 4 and 33°C. The experiment in Table IV indicates that bladders exposed to ADH and fixed with glutaraldehyde at the 0.25% concentration on the mucosa for 5 min retain approximately 74% of the urea permeability of timed controls.

Discussion. Toad bladders fixed on the mucosal surface with 1% glutaraldehyde for 5 min at 21°C following exposure to vasopressin re-

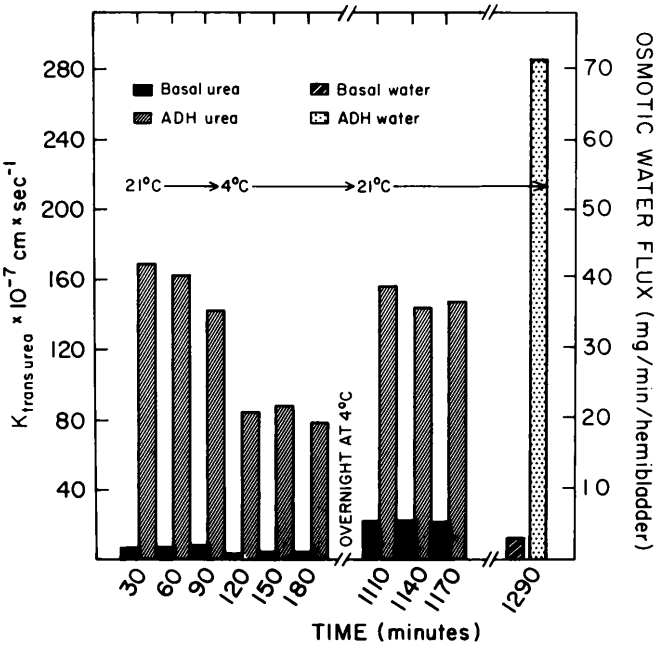

FIG. 1. Preservation of urea and water permeability response to ADH with glutaraldehyde fixation. Matched hemibladders were fixed with 1% glutaraldehyde with (light bars) or without (dark bars) ADH as detailed in Table I. [^{14}C]urea fluxes were studied across the fixed bladders in the absence of hormone at 21°C or 4°C for 20 hr without an osmotic gradient. An osmotic gradient was then established and osmotic water flow measured 1290 min after the bladders had been fixed.

main highly permeable to both water (16) and urea (11) when removed to hormone-free solutions. This phenomenon is illustrated in the experiment shown in Fig. 1, where bladders fixed in the presence of hormone were considerably more permeable to urea and to water than bladders fixed in the absence of hormone even though the fixed bladders had been kept in solutions lacking hormone for more than 20 hr. The present study has demonstrated (Table I) that the vasopressin-sensitive urea permeability barrier can be selectively fixed by lowering the concentration of glutaraldehyde in the mucosal solution from 1 to 0.25%. Bladders fixed with low-dose glutaraldehyde in the presence of hormone remain highly permeable to tracer urea, whereas the characteristic increase in permeability to

TABLE III. TEMPERATURE DEPENDENCE OF VASOPRESSIN-STIMULATED [^{14}C]UREA TRANSPORT ACROSS GLUTARALDEHYDE-FIXED BLADDERS

Time (min)	Temp. (°C)	Bladders A (No ADH) (P_d([^{14}C]urea) × 10^{-7} cm/sec)	Bladders B (with ADH) (P_d([^{14}C]urea) × 10^{-7} cm/sec)	Bladders B − A (P_d([^{14}C]urea) × 10^{-7} cm/sec)		Activation energy (Kcal/mole)
0–30	4	7 ± 4	87 ± 17	80 ± 14	$P < 0.01$	5.3 ± 1.4
30–60	19	17 ± 6	150 ± 28	133 ± 28	$P < 0.02$	5.5 ± 2.1
60–90	33	27 ± 12	227 ± 38	200 ± 38	$P < 0.02$	5.4 ± 1.7
90–120	19	19 ± 4	151 ± 28	134 ± 34		

Note. Matched hemibladders were fixed with 0.25% glutaraldehyde in the presence or absence of ADH as in Table I. Apparent energies of activation were calculated on ADH-specific urea fluxes, i.e., on the difference in fluxes between hemibladders A and B, according to the Arrhenius relationship: $\ln (P_{d1}/P_{d2}) = -Ea/R(1/T_1 - 1/T_2)$. Values given are the mean ± SD of experiments on six toads.

TABLE IV. EFFECT OF 0.25% GLUTARALDEHYDE ON ADH-INDUCED UREA PERMEABILITY

Time (min)	$P_d([^{14}C]urea) \times 10^{-7}$ cm × sec^{-1}	
	Hemibladders A	Hemibladders B
0–30	129 ± 51	108 ± 40 [FIX]
30–60	242 ± 76	179 ± 66

Note. Hemibladders A and B from different toads were filled with Ringer's fluid containing [^{14}C]urea and suspended at room temperature in Ringer's fluid containing 20 mU/ml ADH. After 30 min the B hemibladders were fixed (FIX) for 5 min on the mucosa with 0.25% glutaraldehyde as detailed in Table I. Tracer urea fluxes were measured across the fixed B hemibladders in the absence of ADH during the 30- to 60-min interval. Values given are the mean ± SD on five toads.

water is largely reversed upon washout of hormone. In addition to enriching the ADH-induced urea permeability sites relative to the hormone-stimulated water permeability sites, bladders fixed with low-dose glutaraldehyde exhibit less shunting of tracer urea through nonspecific pathways. Thus, bladders fixed in the absence of hormone with 1% glutaraldehyde are about fourfold more permeable to tracer urea than are bladders fixed with only 0.25% glutaraldehyde (Table I). Therefore, bladders fixed in the presence of hormone with 1% glutaraldehyde are also more permeable to urea than bladders fixed with only 0.25% glutaraldehyde. Previous studies have shown (11) that bladders retain about 74% of their prefixation permeability to urea when fixed with 1% glutaraldehyde following vasopressin stimulation. Similar results were obtained in the present study with 0.25% glutaraldehyde (Table IV). The apparent energy of activation for tracer urea movement across bladders permeabilized by vasopressin and fixed with 0.25% glutaraldehyde was found to be 5.3 (4–19°C), 5.5 (19–33°C), and 5.4 (33–19°C) kcal/mole measured sequentially at 30-min intervals (Table III). The similarity in these values suggests that the fixed urea permeability sites are stable to temperature changes within this range. It is of interest that the value for the activation energy for tracer urea flux in the glutaraldehyde-fixed preparation is comparable to the value of 3.9 kcal/mole measured by Hays and Leaf (21) in unfixed bladders. Since the activation energy for diffusion of urea in water is 4.5 kcal/mole (22) these experiments suggest that the number of hydrogen bonds broken in facilitating urea movement across the bladder wall was equivalent to the number of hydrogen bonds broken as urea diffuses through bulk water. Thus, the rate-limiting step in urea movement across ADH-stimulated, glutaraldehyde-fixed bladders appears to be diffusion of urea through aqueous pathways. The observation that the ADH-induced increase in urea permeability can be selectively preserved by low-dose glutaraldehyde implies that the major pathway taken by water and by urea are separate, as has already been well established by other studies (8–14).

The mechanism by which glutaraldehyde stabilizes the vasopressin-induced increase in urea permeability is not known. It is possible that glutaraldehyde cross-links components of the urea permeability sites at the apical membrane per se or that it acts more indirectly by inhibiting the machinery responsible for the removal of the urea permeability sites. The study in Table II gives some insight as to where glutaraldehyde may act in the hormone target cell. In that study the effective concentration of glutaraldehyde in the mucosal bath was reduced by a combination of a reduction in actual concentration as well as in the temperature of the fixative so that the urea permeability response to vasopressin was no longer preserved by glutaraldehyde. The same conditions of fixation were then employed with glutaraldehyde added to the serosal surface, and now the urea permeability response to vasopressin was well preserved. Since the hormone-induced urea permeability sites are thought to reside in the apical plasma membrane in close contact to the mucosal fluid, one might have expected the opposite result. Clearly, glutaraldehyde does not stabilize the hormone-induced urea channels by an action on the urinary surface of the apical membrane. The simplest explanation for the greater effectiveness of glutaraldehyde added to the serosal side is that is penetrates more rapidly across the basolateral membrane than it does across the apical plasma membrane, and it is the level of glutaraldehyde in the cytoplasm of the hormone target cell which determines

whether or not the urea channels in the apical membrane are fixed. Acting from an intracellular position, glutaraldehyde could stabilize the hormone-induced urea permeability sites either by cross-linkage of the urea channels at the cytoplasmic surface of the apical membrane or by inhibiting the intracellular machinery responsible for the removal or dispersal of the urea channels.

The observation (Table I) that the urea permeability response to vasopressin is selectively preserved by low-dose glutaraldehyde applied to the mucosal surface might be interpreted to show that the hormone sensitive urea permeability barrier is situated superficially in relation to an underlying hormone-sensitive water permeability barrier along the lines originally developed by Leaf (22). Indeed, the experiment in Table II was carried out to see whether it was possible to selectively fix the water permeability barrier from the serosal surface and the urea permeability barrier from the mucosal side. This was not accomplished. Glutaraldehyde (0.17%, 10 min, 4°C) applied to the serosal surface preserved not only the water permeability response to vasopressin, but also the urea permeability, and when applied to the mucosal surface under the same conditions the fixative preserved neither water nor urea permeability. In other studies (23) it has been shown that the concentration of glutaraldehyde required to preserve the effect of vasopressin on water permeability is quite similar to the concentration of glutaraldehyde required to inhibit vasopressin from inducing a response. Both actions of glutaraldehyde are accomplished with approximately one-sixth the concentration when applied to the serosal surface instead of the mucosal side. The greater sensitivity of the urea permeability response to low mucosal doses of glutaraldehyde are, therefore, most readily explained by assuming that the urea channels are more readily stabilized by intracellular glutaraldehyde than are the water channels.

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