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Glutaraldehyde Fixation Preserves the Permeability Properties of the ADH-Induced Water Channels

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Summary. Unidirectional and net water movements were determined, in frog urinary bladders, before and after glutaraldehyde fixation. Experiments were performed in three experimental conditions: 1) in nonstimulated preparations, 2) after the action of antidiuretic hormone (ADH) and 3) in nonstimulated preparations to which amphotericin B was incorporated from the luminal bath. As previously observed for net water fluxes, the increase in the unidirectional water movement induced by ADH was well preserved by glutaraldehyde fixation. After correction for the effects of unstirred layers and nonosmotic pathways, the observed correlation between the ADH-induced increases in the osmotic (Pf) and diffusional (Pd) permeability coefficients was not modified by the fixative action (before glutaraldehyde: slope 11.19, r: 0.87 ± 0.07; n = 12; after glutaraldehyde: slope 10.67, r: 0.86 ± 0.04, n = 39). In the case of amphotericin B, $\Delta Pf/\Delta Pd$ = 3.08 (r: 0.83 ± 0.08), a value similar to that observed in lipid bilayers or in nonfixed toad urinary bladders. It is concluded that 1) The experimental approach previously employed to study water channels in artificial lipid membranes and in amphibian urinary bladders, can be applied to the glutaraldehyde-fixed frog urinary bladder. 2) Glutaraldehyde fixation does not modify the permeability properties of the ADH-induced water channels. 3) Any contribution of exo-endocytic processes or cell regulatory mechanisms to the observed permeability parameters can probably be excluded. 4) Glutaraldehyde-fixed preparations are a good model to characterize these water pathways.

Key Words water channels · glutaraldehyde fixation · frog urinary bladder · unstirred layers · osmotic and diffusional permeabilities · *Rana esculenta*

Introduction

Antidiuretic hormone (ADH) increases the water permeability of target epithelial barriers and strong evidence indicates that the hormonal action is due to the transfer of water channels [3, 16] from cytoplasmic vesicles into the apical plasma membrane of granular cells [21]. This membrane traffic, induced by ADH, can be at the origin of another experimental observation: glutaraldehyde fixation can "freeze" the epithelial barrier in a "high permeability state" (after ADH) or in a "low permeability state" (before ADH) [4, 14]. Nevertheless, while glutaraldehyde-fixed epithelial tissues have been frequently employed [5, 6, 7, 11, 15], it is until now unknown if the fixative modifies the intrinsic permeability of the water pathway. ADH-induced water channels have been recently characterized in frog [22] and toad [18] urinary bladders, by a careful estimation of the osmotic (Pf) and diffusional (Pd) water permeability coefficients. We have now measured these parameters in frog urinary bladders, before and after glutaraldehyde fixation.

As previously made [17, 18, 22], the experimental approach developed to study water permeability in artificial membranes [9, 29, 30] was applied to frog urinary bladders. In pure lipid bilayers Pf/Pd = 1, after appropriate correction of the unstirred layer effects. The incorporation of polyene antibiotics into the membrane increases both Pf and Pd but now $Pf/Pd = N$, N being a number characteristic of the inserted channel. Amphotericin incorporation (N = 3) has been previously employed to test water permeability measurements in toad urinary bladder [18]. We have now also incorporated amphotericin B to frog urinary bladders previously fixed by glutaraldehyde.

The obtained results show that the permeability properties of the ADH-induced channels are not modified by glutaraldehyde and indicate that fixed preparations are a good model for the biophysical characterization of these water pathways.

Materials and Methods

Frog (*Rana esculenta*) urinary bladders were (vertically) mounted as a flat sheet between two twin-barrel Lucite® cells (Fig. 1). A 10-cm hydrostatic pressure applied on the mucosal bath maintained the tissue against a nylon mesh placed on its serosal surface. The exposed tissure area was twice 1.77 cm^2. The four chambers (mucosal and serosal bath, control and experimental channels) were filled with 5 ml buffer solution (R) con-

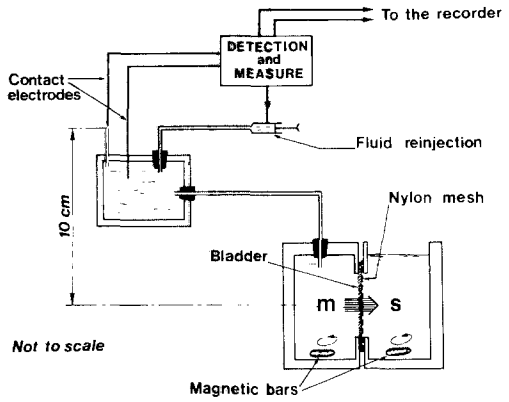

Fig. 1. Schematic representation of the experimental device

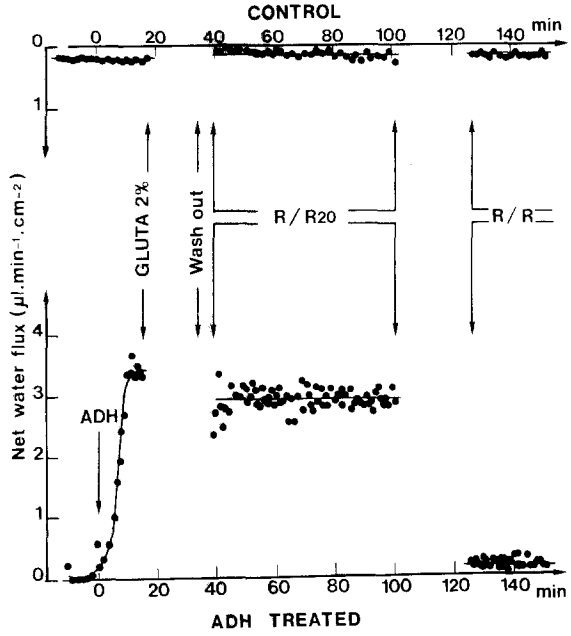

Fig. 2. Effect of glutaraldehyde (GLUTA) fixation on the net water flux, in control conditions and after ADH (oxytocin 2.2×10^{-8} M)

taining (mM): NaCl 111.5; KCl 5.0; $NaHCO_3$ 2.5; $CaCl_2$ 1.0; pH 8.1 ("*R*/*R*" in tables and figures indicates that the same solution (*R*) was present in the serosal and mucosal chambers). When an osmotic gradient was applied, NaCl concentration was reduced to 5.2 mM in the mucosal bath ("R/R_{hypo}"). Mixing was ensured by four magnetic bars, turning at a constant rate through all experiments.

Net Water Measurements

The net water transfer was measured with the technique of Bourguet and Jard [1]: Water was injected into the mucosal chamber to maintain a constant volume. The amount injected every minutes, and equivalent to the net flux, was automatically recorded. At the end of a measurement period performed in the presence of an osmotic gradient, the net water flux was again estimated with both the mucosal and serosal chambers filled with isosmotic solutions. The osmotic permeability coefficient (Pf) was estimated by subtracting the permeability corresponding to the "nonosmotic net flux" (driven by the applied hydrostatic pressure), from the osmotic permeability coefficient observed in the presence of an osmotic gradient (Pf_{obs}).

Table 1. Effect of glutaraldehyde fixation on the observed net water fluxes

	Net water fluxes ($\mu l \cdot min^{-1} \cdot cm^{-2}$, R/R_{hypo})	
	Before fixation	After fixation
Nonstimulated ($n = 8$)	0.12 ± 0.01	0.27 ± 0.03
Oxytocin, 2.2×10^{-8} M ($n = 8$)	2.39 ± 0.15	1.87 ± 0.12

Unidirectional Water Fluxes

The unidirectional water transfer was estimated from ^{3}HHO fluxes in the absence of an osmotic gradient, as previously described [24]. The radiotracer (2 μC/ml) was added to the mucosal chamber at the beginning of the experiment. All the serosal volume was aspirated every two minutes and replaced by cold buffer. The ^{3}HHO transfer was measured in at least 15 consecutive periods, by scintillation counting, and the water permeability coefficient (Pd_{obs}) determined.

The thickness of the unstirred layers (d) in series with the tested membrane was estimated from ^{14}C-butanol permeability experiments [9, 13]:

$$d = D/P_{but} \quad (1)$$

where D is the diffusion coefficient for butanol in water and P_{but} the observed butanol permeability. In 10 different experiments performed in our experimental conditions we obtained for the unstirred layer thickness a value of 442 ± 29 μm. This figure was used to correct the observed diffusion permeability coefficient Pd_{obs} according to [10, 12, 30]

$$1/Pd_{obs} = 1/Pd' + d/Do \quad (2)$$

where Pd' is the water diffusional permeability coefficient after correction for the unstirred layer effects and Do the diffusion coefficient for water in water.

The existence, in our experimental conditions, of "nonspecific leaks" was estimated from ^{14}C-sucrose permeability experiments [28]. This radiotracer was added (1 μC/ml) to the mucosal bath, together with ^{3}HHO, in most unidirectional flux experiments. Appropriated corrections for double marking were performed to calculate sucrose and water fluxes. Assuming that water moves together with sucrose across this "nonosmotic pathway" and that the ^{3}HHO/^{14}C-sucrose specific activity ratio remains constant all the way, the amount of water moving through this route can be easily calculated, as well as its water diffusional permeability (Pd_s). Finally, the water diffusional permeability (Pd) can be obtained from:

Table 2. Permeability parameters measured in glutaraldehyde-fixed frog urinary bladder[a]

Fixation condition	n	$Jv\ R/R_{hypo}$	$Jv\ R/R$	Pf_{obs}	Pf	Jd	Pd_{obs}	Pd_s
Nonstimulated	16	0.31 ±0.04	0.05 ±0.02	14.06 ±1.98	11.63 ±1.89	10.96 ±0.54	1.80 ±0.09	30.72 ±5.26
Oxytocin 2.2×10^{-8} M	24	1.98 ±0.12	0.16 ±0.02	91.25 ±5.45	83.79 ±5.55	18.16 ±0.57	3.00 ±0.10	47.07 ±4.64

[a] $Jv\ R/R_{hypo}$: Net water flux measured in the presence of an osmotic gradient. $Jv\ R/R$: Net flux measured in the absence of an osmotic gradient. Jd: Unidirectional water flux (no osmotic gradient). (Water fluxes in $\mu l \cdot min^{-1} \cdot cm^{-2}$). Pf_{obs}: Observed osmotic permeability coefficient ($cm \cdot sec^{-1} \times 10^4$). Pf: Osmotic permeability coefficient after correction for nonosmotic fluxes ($cm \cdot sec^{-1} \times 10^4$). Pd_{obs}: Observed diffusional permeability coefficient ($cm \cdot sec^{-1} \times 10^4$). Pd_s: Observed sucrose permeability coefficient ($cm \cdot sec^{-1} \times 10^7$).

$$Pd = Pd' - Pd_s. \quad (3)$$

We will analyze in the Addendum section the validity of employing butanol permeability measurements to estimate the "equivalent" unstirred layers for water in frog urinary bladder. The term ADH is used through the text as a generic expression covering the hydrosmotic action of oxytocin (the hormone employed in this work) and other neurohypophyseal peptides.

Results

Effect of Glutaraldehyde Fixation on the ADH-Induced Hydrosmotic Response

Figure 2 shows a typical experiment. Two fragments of the same bladder were studied (*see* Materials and Methods) in the presence of an osmotic gradient. Oxytocin (2.2×10^{-8} M) was added to the experimental channel and at the maximum of the hydrosmotic response both the control and experimental fragments were fixed by glutaraldehyde addition (2% in buffer solution) during 20 min. The fixative was then washed and the net water flux measured again. It can be observed that while the osmotic permeability of the control fragment remained at the same low value, the response to oxytocin was almost fully preserved in this case. In most cases, however, the basal flux increased (up to twofold the reference value), while the response to oxytocin was in average reduced to 78% of the control (Table 1).

Net and Diffusional Water Fluxes Before and After Glutaraldehyde Fixation

Two fragments of the same bladder were fixed, as previously described, one in control conditions and the other at the maximum of the hydrosmotic response to oxytocin. The net water fluxes were recorded, in the presence of an osmotic gradient ($Jv\ R/R_{hypo}$). Next the mucosal chambers were open and all solutions replaced by isosmotic buffer. Unidirectional water fluxes (Jd) and sucrose permeabilities (Pd_s) were then measured from the ^{3}HHO and ^{14}C-sucrose fluxes, as described in Materials and Methods. Finally, the net water fluxes were measured again, now in the absence of an osmotic gradient ($Jv\ R/R$). The observed results are presented in Table 2. In the absence of an osmotic gradient ($Jv\ R/R$) a relatively small net water flux appeared, not observed in nonfixed bladders. This "nonosmotic pathway" represented less than 10% of the total water flow.

Table 3. Permeability parameters measured in nonfixed frog urinary bladders[a]

Fixation condition	n	$Jv\ R/R_{hypo}$	Pf	Jd	Pd_{obs}	Pd_s
Nonstimulated	13	0.11 ±0.001	5.23 ±0.56	9.45 ±0.94	1.56 ±0.17	6.17 ±1.90
Oxytocin 2.2×10^{-8} M	6	2.39 ±0.15	108.0 ±5.42	20.22 ±1.00	3.36 ±0.17	3.99 ±0.56

[a] Notes as in Table 2. $Jv\ R/R$ (*not shown*) was not significantly different from zero.

Osmotic and diffusional permeabilities have been previously measured in nonfixed frog urinary bladders [22]. Table 3 gives the results obtained with the here described experimental approach. When the obtained values are compared to those reported in Table 2 it is observed that glutaraldehyde fixation induced a six- to 10-fold increase in sucrose permeability.

The $\Delta Pf/\Delta Pd$ Ratio in Fixed and Nonfixed Frog Urinary Bladders

Pd_{obs} and Pf_{obs} values, corrected for the effects of unstirred layers and nonspecific pathways, give a

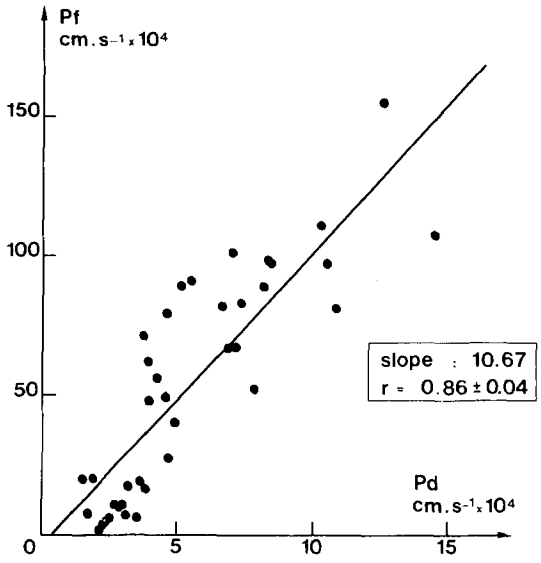

Fig. 3. Observed correlation between the osmotic (*Pf*) and diffusional (*Pd*) permeability coefficients in glutaraldehyde-fixed frog urinary bladders. Values from ADH-stimulated and nonstimulated preparations, after correction for unstirred layers and nonosmotic pathways (*see* Materials and Methods)

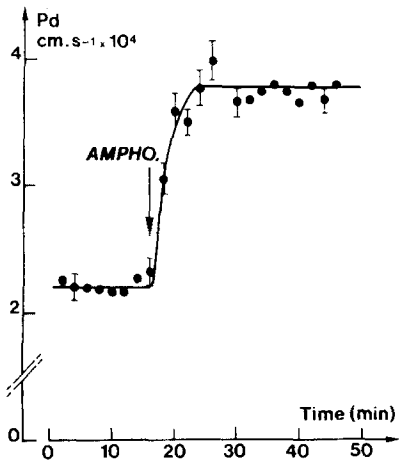

Fig. 4. Effect of amphotericin B (*AMPHO*) addition (12.5 $\mu g \cdot ml^{-1}$) on the observed unidirectional water movement in frog urinary bladders previously fixed with glutaraldehyde

series of paired values for *Pf* and *Pd*. Figure 3 is a correlation plot between these two parameters in the case of fixed preparations (including both stimulated and nonstimulated preparations). The slope of the regression line was 10.67 ± 2.34 with a correlation coefficient of 0.86 ± 0.04 (n = 39). The same was done with the results obtained with nonfixed bladders and the observed slope was 11.19 with a correlation coefficient of 0.87 ± 0.07 (n = 12).

Incorporation of Amphotericin B Channels in Frog Urinary Bladders Fixed in Nonstimulated Conditions

Amphotericin B addition to the mucosal chamber (12.5 μg/ml) increased the water permeability of previously fixed frog urinary bladders (Fig. 4). The results observed in six different experiments for the modifications in the water net (*Jv*) and unidirectional (*Jd*) fluxes, as well as sucrose permeabilities (Pd_s) were treated as described in Materials and Methods. Figure 5 shows a correlation plot between the calculated paired values for *Pf* and *Pd*. The observed slope ($\Delta Pf/\Delta Pd$) was 3.08 with a correlation coefficient (r) of 0.83.

Effects of CO_2 Bubbling, After Glutaraldehyde Fixation, on the ADH-Induced Water Permeability

Cellular acidification rapidly reduces the ADH and cyclic AMP-induced net water fluxes in situations in which the ADH-induced water channels seem still to be in the apical membrane [23, 25]. We have now tested the effects of CO_2 bubbling (5% in the serosal side, medium pH dropped to 6.5) on the hydrosmotic response induced by 8-bromo-cyclic AMP. Figure 6 shows that, after glutaraldehyde fixation, sensitivity to medium acidification was lost.

Discussion

While the effect of glutaraldehyde fixation on the ADH-induced increase in net water fluxes is well documented [4–7, 14], the action of this fixative on the unidirectional water permeability has not been previously studied. We have now measured both parameters in fixed and nonfixed preparations, and after the obtained results, several points seem to be clear:

1) Amphotericin B still incorporates into the apical membrane after fixation and the observed ratio between the increases in *Pf* and *Pd* was ≈3, as in the case of artificial systems. This result strongly supports the validity of the employed correction methods.

2) We report here a $\Delta Pf/\Delta Pd$ value, after ADH, of about 11 in fixed frog urinary bladders. In nonfixed preparations we obtained practically the same value. These figures can be compared with the previous ones reported for frog urinary bladder ($9 < \Delta Pf/\Delta Pd < 18$ [22]) and toad urinary bladder ($\Delta Pf/\Delta Pd \approx 17$ [18]). The biophysical significance of these numbers has been previously evaluated [18, 22] and we will not discuss this point again. Nevertheless, it must be mentioned that a similar result ($\Delta Pf/\Delta Pd \approx 11$) has been recently reported for the red cell water channel [20]. In this case the applied

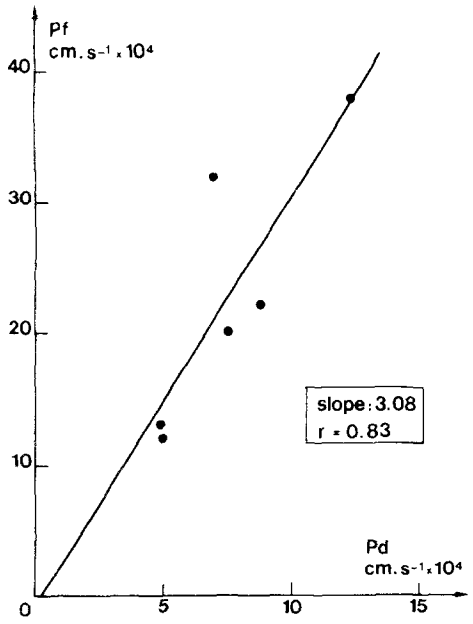

Fig. 5. Observed correlation between the osmotic (*Pf*) and diffusional (*Pd*) permeability coefficients in glutaraldehyde-fixed frog urinary bladders. Values were determined after amphotericin B addition to the mucosal bath

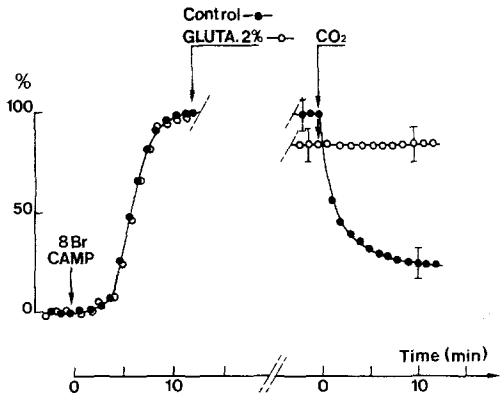

Fig. 6. Effect of CO_2 bubbling (CO_2 5%, serosal side, pH 6.5) on the 8 Br-cyclic AMP-induced net water flux in fixed (*GLUTA*, open circles) and nonfixed (closed circles) urinary bladders

rationalism was identical to the one employed here but the experimental approach was completely different. If these figures are confirmed, it can be concluded that ADH action plugs into the apical membrane water channels that are functionally similar to the ones observed in red cells.

3) After glutaraldehyde fixation, both the ADH-induced net and unidirectional fluxes were reduced (Table 1) but $\Delta Pf/\Delta Pd$ remained constant, suggesting that glutaraldehyde fixation did not modify the permeability properties of the ADH-induced water channels. The proportional reduction in *Pf* and *Pd* can be understood if we accept that during the fixation procedure some permeability units are lost while the remaining ones maintain their permeability properties. This would be possible if the cross-linking reagent interacts with chemical groups outside the channel.

Under certain conditions, cellular acidification rapidly reduces water permeability while the water channels are still present in the apical membrane [23, 25]. These results have been interpretated as a consequence of a direct effect of low cellular pH on the water channel, that would shift from an "open" to a "closed" state. Alternatively, it has been suggested that cellular acidification induces the appearance of a cytoplasmic barrier in series with the channels [23]. If the first hypothesis is accepted, the loss of reactivity to changes in intracellular pH would indicate that some "rigidity" was introduced by the fixative action on the channel structure. To accept the second possibility, it will be necessary to propose that the pH-induced change in the cytoplasm structure is inhibited by glutaraldehyde.

A direct demonstration of complete fixation is difficult to experimentally approach. Nevertheless, experiments with fixed tissues are important (the glutaraldehyde capacity to "freeze" a permeability pathway in two different states is not restricted to the ADH-induced water channels [27]) because any possible contribution of cell metabolism, exo-endocytic processes or cell regulatory mechanisms will be drastically reduced. The presence of an important net water flux can modify water permeability, in frog urinary bladder, via a metabolically dependent, negative regulatory feed-back [6, 8, 26]. Because of this situation comparable osmotic and diffusional studies are difficult to perform [22, 26]. This problem is circumvented in fixed preparations, opening the road to experiments in which the role of osmotic gradients can be evaluated independently of any permeability effect.

Freeze-fracture studies have given strong support to the "channels-transfer" hypothesis. Interestingly enough, most freeze-fracture studies are performed after glutaraldehyde fixation. Present results, indicating that water channels are fully operative in this condition, give more credibility to studies correlating structural and permeability properties.

Addendum

Validity of the Employed Correction to Estimated *Pd* and *Pf*

It has been considered that it would be inappropriate to use butanol (or other lipophilic molecule) to measure the barriers in series for water movement across a tissue, because the resistance offered to a lipophilic molecule would not be the same, inside the cell, as that offered to water [13, 17]. In fact the un-

stirred layers in series with the luminal membrane can be divided in two different parts: the "intra-tissular" and "extra-tissular" barriers. We have observed, in our experimental conditions, a "water equivalent thickness" for the barrier to butanol diffusion of about 440 μm. Levine et al. [17] estimated that the toad bladder is equivalent to a 99-μm-thick unstirred aqueous layer. In frog urinary bladder this figure must be still smaller, because this tissue has a much less developed supporting layer than toad bladder [2]. It can be concluded that the extra-tissular unstirred layers must be the most important ones in our experimental conditions. This situation can explain why corrections from methanol [22] or butanol (present experiments) allow as a good "rectification" [9, 22] of the $\Delta Pf/\Delta Pd$ curve. This good linearity (Fig. 3 and Figs. 2 and 3 in Ref. [22]) is one of the arguments we (and others, [18]) have to accept the validity of the employed correction method. A second and important support arises from amphotericin B experiments: unstirred layer thickness obtained from butanol experiments were also employed to correct the observed *Pd* values.

Because of physical intracellular constraints, other than the mucosal border permeability, *Pf* values can be underestimated when important water movements are observed [19]. We have previously analyzed that, in extreme conditions, this situation could imply a doubling of the *Pf* value and then also a doubling of the *Pf/Pd* ratio [22]. Consequently, and putting together present and previous results [22], it can be accepted that $11 < \Delta Pf/\Delta Pd < 18$ for the ADH-induced water channels in frog urinary bladder. Incertitudes arising from the complex arrangement of barriers in series and parallel preclude a sharper estimation of this parameter, at least in our experimental conditions.

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