J. Membrane Biol. 71, 189–193 (1983)

The Journal of
Membrane Biology

# The Single File Hypothesis and the Water Channels Induced by Antidiuretic Hormone

Mario Parisi and Jacques Bourguet
Département de Biologie, CEN Saclay, 91191 Gif sur Yvette, France

**Summary.** Unidirectional and net water movements were determined at minute intervals in frog urinary bladders. The changes in both parameters were followed, during the action of antidiuretic hormone (ADH), at different temperatures and stirring conditions. After correction for external unstirred layer effects, the ratio of the osmotic ($Pf$) and diffusional ($Pd$) permeability coefficients was remarkably constant, at different times and in different experimental conditions. In the presence of ADH the $\Delta Pf/\Delta Pd$ ratio in the mucosal border was probably greater than 9. On the other hand, in nonstimulated preparations the ratio was smaller, and probably not different from 1. These results, together with previous observations indicating that other small molecules (like urea) are excluded from the ADH-induced channel, might indicate that single-file water movement can occur through this structure. Alternatively, the $\Delta Pf/\Delta Pd$ ratio could result from a complex geometric arrangement in series with the aqueous pore.

**Key words** water channels · antidiuretic hormone · frog urinary bladder · unstirred layers · osmotic permeability · diffusional permeability

## Introduction

In membranes containing aqueous channels the osmotic permeability coefficient ($Pf$) is greater than the diffusive permeability coefficient ($Pd$). The $Pf/Pd$ ratio has been carefully studied in the case of lipid bilayers treated with gramicidin-A and other polyene antibiotics [11, 19]. These results suggest (after appropriate correction for unstirred layer effects) that single-file water transport can occur in those membranes, the $Pf/Pd$ ratio indicating the number of water molecules inside the channels.

ADH-induced pores hold back urea [8, 13], thus implying a pore radius smaller than 2 Å, as in the case of gramicidin. It has been suggested [10, 19] that single-file transport could also occur through them. We have recently described an experimental approach that allows the simultaneous determination of net and unidirectional water fluxes in epithelial barriers [15]. Applying the previously reported approach [19] to tracer diffusion and osmotic experiments, performed in different conditions, we have now estimated the $Pf/Pd$ ratio, after correction for external unstirred layer effects, in frog urinary bladders under oxytocin. Because of the complexity of the tested structure, the relationship obtained can indicate either the number of water molecules inside the channel or the existence of more complex geometric arrangements in series with each aqueous pore.

## Materials and Methods

Frogs (*Rana esculenta*) were kept at 20 °C in running tap water for at least 5 days before the experiment. The bladders were removed from pithed frogs and mounted between two Lucite chambers.

Unidirectional water fluxes were measured as previously described [15]: The bladder was disposed horizontally, with the mucosal border facing the lower chamber, and a nylon mesh placed on its upper side. The volume of the lower compartment was 12 or 7 ml, and the volume of the upper one was 2.0 ml. Both solutions could be vigorously stirred with magnetic bars (stirring was less effective with the smaller chamber). $^3$HOH was added to the lower bath up to a final concentration of 10 μCi/ml. The solution in the upper chamber was then completely removed every minute and refilled with unlabeled solution. The $^3$HOH activity of the sample was determined and the unidirectional flux expressed in $\mu l \cdot h^{-1} \cdot cm^{-2}$ (the exposed area was 3.1 or 1.6 $cm^2$ in different experiments). The specific activity in the inferior chamber was recalculated for each period, taking into account the previous transfer of radioactivity [15]. $^{14}$C-methanol unidirectional fluxes were simultaneously determined in some experiments.

The net flux of water was simultaneously measured with a previously described technique [3]: In the presence of an osmotic gradient (mucosal side hypotonic), net water flux proceeded from the inferior to the superior solution. Accordingly, water was injected into the lower chamber to maintain constant volume. The amount of water injected every minute, and equivalent to the net flux, was recorded.

The methanol molecule is very soluble in lipids and cholesterol, and its measured permeability was strongly dependent on stirring conditions. We can accept, as previously found in the case of butanol [11] or other molecules soluble in organic

0022-2631/83/0071-0189 $01.00

media [10], that the resistance to methanol diffusion across the cellular membrane was negligible compared to the resistance of the in-series unstirred layer. If this is true, the unstirred layer thickness can be calculated from [10, 11]

$$1/P_{meth} = \delta/D \quad (1)$$

where $P_{meth}$ is the observed methanol permeability, $\delta$ the unstirred layer thickness and $D$ the diffusion coefficient for methanol in water ($10^{-5}$ cm$^2$·sec$^{-1}$). The observed $P_{meth}$ at 20 °C were 3.57 and $1.17 \times 10^{-4}$ cm·sec$^{-1}$ for the 3.1 and 1.6 cm$^2$ surface chambers. Applying Eq. (1) the calculated unstirred layer was 280 μm when the larger chamber was used and 850 μm with the smaller one.

The serosal bath composition was (in meq/liter): $Na^+$, 114.5; $Ca^2$, 1.0; $K^+$, 5.0; $Cl^-$, 110.0; $HCO_3^-$, 2.5; pH 8.1. NaCl concentration was reduced to 5.6 mM in the mucosal solution during osmotic challenge. Experiments were performed either at 20±1 or at 9±1 °C.

## Results and Discussion

After oxytocin addition ($2.2 \times 10^{-8}$ M) both unidirectional and net water fluxes increased, following a sigmoidal function with time [15]. Figure 1 shows the mean curves (six experiments in each case) for the evolution of unidirectional and net water fluxes in two experimental conditions: at 20 °C, employing the 3.1 cm$^2$ chamber, and at 9 °C, with the 1.6 cm$^2$ one. At 9 °C both parameters increased more slowly, as previously described [1, 16]. Furthermore, and probably because a combined action of cold and thicker unstirred layers, the observed increase in the unidirectional flux was proportionally smaller than the one observed when employing the 3.14 cm$^2$ chamber. As previously reported [15], no differences were observed between the increases in the unidirectional fluxes in the presence or absence of an osmotic gradient.

The Table gives (columns 2 and 7, columns 3 and 8) the observed diffusive permeability coefficient ($Pd_{obs}$) and the osmotic permeability coefficient ($Pf$) calculated, in the two tested conditions, from the corresponding unidirectional and net water fluxes (Fig. 1).

### *Evaluation of Unstirred Layer Effects in Planar Bilayers and Epithelial Barriers*

It would be of interest to determine the real value for the $Pf/Pd$ ratio, before and after ADH stimulation, in the apical border of granular cells of amphibian urinary bladder. Different attempts have been made to estimate this ratio, by measuring the transepithelial water permeability. Nevertheless, uncertainties arising from unstirred layer effects made it difficult to estimate the mucosal border permeability [5, 6, 9]. The main purpose of this work is to apply and discuss the validity of a method developed to treat unstirred layer effects in doped bilayers [19] to pairs of values for diffusional and osmotic water permeability simultaneously determined in frog urinary bladders. These parameters were measured during ADH stimulation. We have recently proposed that the observed increase in water permeability results from the addition of channels (represented by the intramembranous particle aggregates appearing in the presence of ADH [4, 12] that increase in number during the development of the hormonal action [15]. We are then in a situation similar to the one studied when $Pf$ and $Pd$ values are measured in artificial bilayers, as a function of the number of gramicidin channels.

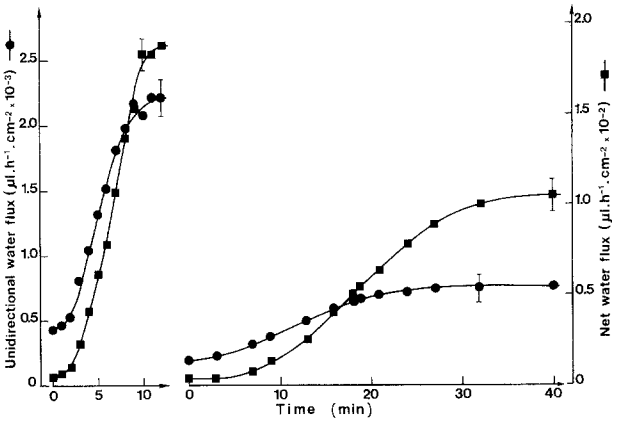


**Fig. 1.** Time course evolution of the observed unidirectional (dots) and net (squares) water fluxes under oxytocin, at 20 °C (left) and at 9 °C (right). The hormone ($2.2 \times 10^{-8}$ M was added at zero time. For simplicity, only some of the minute-by-minute values obtained were represented at low temperature. Mean±SE for six experiments in each case

### *Influence of Unstirred Layer on the Observed Diffusional and Osmotic Permeabilities*

Whereas $Pf$ generally does not depend on the stirring rate of the medium, and this is the case in our experimental conditions [15], $Pd_{obs}$, on the contrary, is strongly influenced by unstirred layers [9, 15]. In toad urinary bladder values of about $1.5 \times 10^{-4}$ and $6 \times 10^{-4}$ cm·sec$^{-1}$ have been reported for water diffusional permeability in the presence of slow or vigorous medium stirring, respectively. These figures can be compared with the present value of about $6 \times 10^{-4}$ cm·sec$^{-1}$, obtained at 20 °C and under vigorous stirring conditions (Table, column 2, last line). Nevertheless, unstirred layers can never be completely removed, especially those represented by the bladder tissue itself. To take into account the effect of unstirred layers, it is generally accepted that the "correct"

**Table.** $Pd$ and $Pf$ values in two different experimental conditions and at different times after oxytocin addition ($2.2 \times 10^{-8}$ M)[a]

| 20 °C, Larger chamber | | | | | 9 °C, Smaller chamber | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $t$ (min) | $Pd_{obs}$ | $Pf$ | $Pd_m$* | $\Delta Pf/\Delta Pd_m$* | $t$ (min) | $Pd_{obs}$ | $Pf$ | $Pd_m$** | $\Delta Pf/\Delta Pd_m$** |
| 0 | 1.16 | 3.1 | 1.32 | – | 0 | 0.51 | 2.8 | 0.62 | – |
| 1 | 1.26 | 4.5 | 1.45 | 10.7 | 3 | 0.61 | 2.9 | 0.79 | 17.0 |
| 2 | 1.46 | 7.4 | 1.72 | 10.7 | 7 | 0.86 | 5.7 | 1.26 | 8.3 |
| 3 | 2.26 | 17.4 | 2.95 | 8.8 | 9 | 1.05 | 10.0 | 1.72 | 6.5 |
| 4 | 2.92 | 31.8 | 4.19 | 10.0 | 13 | 1.39 | 19.9 | 2.89 | 7.5 |
| 5 | 3.67 | 47.6 | 5.93 | 9.6 | 16 | 1.62 | 31.3 | 4.09 | 8.2 |
| 6 | 4.22 | 60.5 | 7.52 | 9.2 | 18 | 1.76 | 39.3 | 5.12 | 8.2 |
| 7 | 5.07 | 83.3 | 10.72 | 8.8 | 19 | 1.83 | 42.7 | 5.76 | 7.8 |
| 8 | 5.52 | 106.3 | 12.96 | 8.8 | 21 | 1.93 | 49.8 | 6.89 | 7.5 |
| 9 | 6.08 | 119.2 | 16.50 | 7.6 | 24 | 1.98 | 61.2 | 7.57 | 8.4 |
| 10 | 5.77 | 142.2 | 14.40 | 10.6 | 27 | 2.08 | 69.8 | 9.28 | 7.7 |
| 11 | 6.18 | 143.6 | 17.30 | 8.8 | 32 | 2.09 | 78.3 | 9.48 | 8.5 |
| 12 | 6.18 | 146.2 | 17.30 | 9.0 | 40 | 2.12 | 81.2 | 10.13 | 8.2 |
| | | | Mean | 9.38 | | | | Mean | 8.65 |
| | | | SEM | 0.27 | | | | SEM | 0.32 |

[a] Mean of 6 experiments in each case ($cm \cdot sec^{1} \cdot 10^{4}$).

$Pd_{obs}$: Observed values for the diffusional permeability ($Pd$). $Pd_m$:* Estimated values for the limiting membrane $Pd$ assuming the unstirred layer thickness ($\delta$) = 250 μm. $Pd_m$**: Estimated values for the limiting membrane $Pd$ assuming $\delta$ = 900 μm. $\Delta Pf = Pf(t) - Pf(0)$; $\Delta Pd_m{}^* = Pd_m{}^*(t) - Pd_m{}^*(0)$; $\Delta Pd_m{}^{**} = Pd_m{}^{**}(t) - Pd_m{}^{**}(0)$.

value for the diffusional permeability can be calculated from [7]

$$1/Pd_{obs} = 1/Pd_m + \delta/D \quad (2)$$

where the membrane permeability ($Pd_m$) is obtained from the observed permeability ($Pd_{obs}$), the unstirred layer thickness ($\delta$) and the diffusion coefficient for water in water ($D$, $2.4 \times 10^{-5}$ $cm^2 \cdot sec^{-1}$).

The lower curve in Fig. 2 represents the $Pd_{obs}$ values as a function of $Pf$ at 20 °C. Its progressive bending is probably due to the more and more important effect of unstirred layers on $Pd_{obs}$ as the membrane permeability increases (in the absence of unstirred layers a linear relationship between $Pf$ and $Pd$ is expected). The type of relationship between $Pf$ and $Pd_{obs}$ presented in Fig. 2 is quite similar to the one observed in the case of planar bilayers doped with gramicidin [18]. This encouraged us to apply the same treatment to take into account the effect of unstirred layers.

When the $Pd_{obs}$ values obtained at 20 °C were corrected by applying Eq. (1), a complete rectification was obtained if $\delta$ was taken as = 250 μm (Fig. 2). This unstirred layer thickness is quite similar to the figure obtained from methanol experiments (280 μm). It can be remarked that values of $\delta$ of 200 or 350 μm clearly caused deviation from the linear relationship. Column 4 in the Table presents the $Pd_m$ values calculated with Eq. (2), using $\delta$ = 250 μm and the corresponding $Pd_{obs}$. After rectification, the ratio between the increases in $Pf$ and $Pd_m$ induced by the hormone ($\Delta Pf/\Delta Pd_m$) becomes ≃ 9 ($\Delta Pf$ and $\Delta Pd$ are the differences between the permeability values at different times after oxytocin stimulation and at zero time; *see* Table).

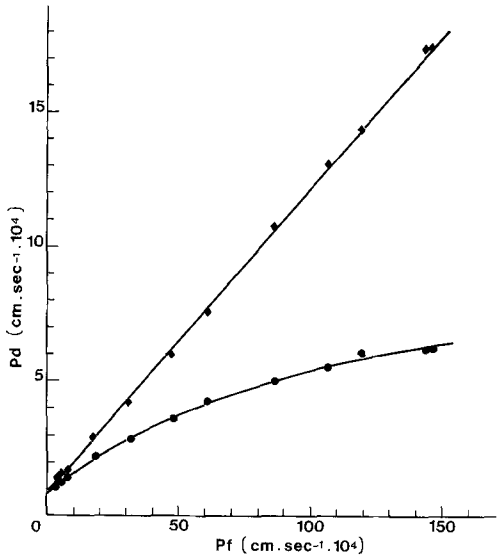


**Fig. 2.** Lower curve: Observed values ($Pd_{obs}$) for $Pd$ as a function of the simultaneously measured osmotic permeability ($Pf$) at different times after oxytocin addition ($t°$ = 20 °C, high stirring rate). *Upper curve:* Corrected values ($Pd_m$) applying Eq. (2) and assuming $\delta$ = 250 μm

This figure was confirmed by experiments performed in completely different experimental condi-

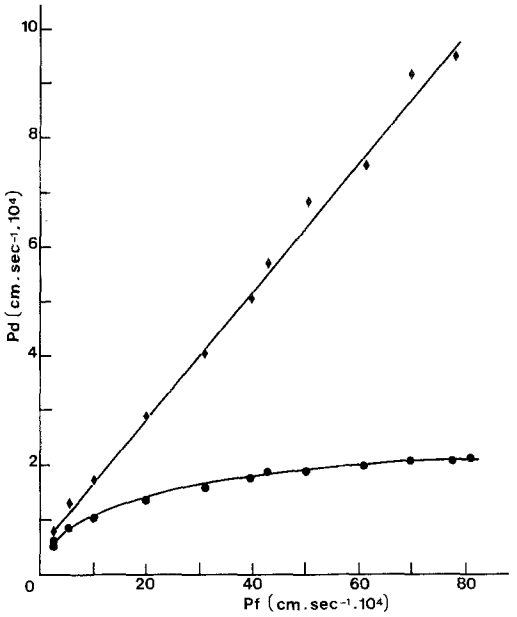


**Fig. 3.** Lower curve: $Pd_{obs}$ as a function of $Pf$ at different times after oxytocin addition ($t° = 9$ °C, low stirring rate). Upper curve: Corrected values for $Pd_m$ applying Eq. (2) and assuming $\delta = 900$ μm

tions. The lower curve in Fig. 3 represents the $Pd_{obs}$ values as a function of $Pf$ during the response to oxytocin at 9 °C (smaller chamber). In this case the unstirred layer must be larger, as suggested by methanol experiments. To obtain complete rectification in this series, a 900 μm unstirred layer was employed (Fig. 3). Interestingly enough, this value was again similar to the one calculated from methanol experiments, but the more exciting point was that we had again, after rectification, $\Delta Pf/\Delta Pd_m \simeq 9$. Column 9 in the Table gives the $Pd_m$ values calculated with Eq. (2), employing the corresponding $P_{obs}$ values and taking $\delta$ as equal to 900 μm. Columns 5 and 10 in the Table show that we obtain the same relationship, after correction for unstirred layers effects, in two series of experiments performed in different physiological and stirring conditions.

It must be considered here that $Pd$ values are generally calculated from $^3$HOH fluxes measured in the absence of an osmotic gradient. However, in a previous paper [15] we have shown that there are no significant differences between the time-course of the water permeability response to ADH in the presence or absence of an osmotic gradient. This observation was now confirmed, and because it was essential that $Pd$ and $Pf$ were simultaneously measured, we have accepted that the $Pd_{obs}$ value obtained from unidirectional measurements performed in the presence of an osmotic gradient is a good estimation of the diffusional permeability. In any case the eventual error (a few percent variation) will not significantly change the $Pf/Pd$ ratio.

*Validity of the Pd Value after Correction for Unstirred Layer Effects*

To accept that the $Pd_m$ value obtained after correction for unstirred layers represents the $Pd$ value for the mucosal border, it must be assumed that the bladder tissue, other the apical membrane, can be considered as a part of the unstirred layers. The thickness of the tissue (about 20 μm) represents less than 5% of the calculated unstirred layer thickness, but it is difficult to assert that it does not represent a more complex barrier to water (and methanol) diffusion than a similar (or thicker) layer of water (*see* ref. 10). It is interesting to observe that a "restricted diffusion," in the sense of a smaller diffusion coefficient for water inside the cell, will not invalidate the employed method. Furthermore, if the restriction is similar for water and methanol, it would not be unexpected that both methods give the same values. Nevertheless, this value will not represent the real unstirred layer thickness but an equivalent one, valid to obtain $Pd_m$. We are planning future experiments to clarify this controversial point.

*Validity of the Obtained Pf Value to Estimate the Osmotic Permeability of the Mucosal Border*

The main assumption behind the unstirred correction method previously employed is that the mucosal border represents the only significant barrier to net water movement. It has been recently reported that, in sac preparations, the $Pf$ values could be underestimated because physical constraints, other than the mucosal border permeability, when important net water movements are observed [14]. Consequently, the figure obtained for $\Delta Pf/\Delta Pd$ must be considered a minimum value. In fact, the net water flux induced by ADH in the presence of an osmotic gradient induce cell swelling and dilution of the intracellular fluid. This evidently reduces the osmotic gradient across the mucosal border and $Pf$ will be proportionally underestimated. When the mucosal border is exposed to a very diluted media the granular cells roughly doubled their volume. Consequently, the cell tonicity will drop to one half. This would signify, in this situation, a doubling of the $Pf$ value and also a doubling of the $Pf/Pd$ ratio.

*The Pf/Pd Ratio and the Single File Hypothesis*

It seems reasonable to conclude from our results, that the $Pf/Pd_m$ ratio is greater than one, after

correction for unstirred layer effects, in the frog urinary bladder stimulated by ADH; earlier experiments on unstirred layers in frog skin [6] or renal collecting duct [10] also suggested this. Furthermore, the $Pf/Pd_m$ ratio would have a minimum value of 9, if underestimation of $Pf$ because of intracellular constraints to net water movement are taken into account. The fact that the $Pf/Pd$ ratio exceeds unity together with the observation that the water channel excludes small molecules like urea [13] gives support to the hypothesis proposing a single-file mechanism for water movement across the ADH-induced channel. It could be also interpreted, following the treatment previously developed for the gramicidin channel by Rosenberg and Finkelstein [18], that the $Pf/Pd$ ratio gives the number of water molecules present in the ADH-induced pore. Nevertheless, it must be kept in mind that this conclusion is based on all the previously discussed assumptions. Perhaps Eq. (2) should not be used to correct for "intracellular" unstirred layers. A simple geometrical arrangement is implicit when this equation is used, and it would not be unexpected that this is not the case in epithelial barriers [10, 15]. It should also be said that while the gramicidin channel seems to accept a single-file movement for ion transport [18, 20] this is not the case for the ADH-induced channel [10].

We have also observed that the $Pf/Pd$ ratio shows, in the absence of ADH, a much smaller value, probably not significantly different from unity. This might indicate that the mechanism for water permeation "at rest" is different from the one induced by the hormonal stimulation. This is in agreement with morphological and physiological observations suggesting that ADH induces the appearance of a new water pathway [2, 4, 12, 17].

We wish to express gratitude to Prof. C.A. House, from the University of Edinburgh, who has critically read the manuscript.

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Received 13 April 1982