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SIMULTANEOUS MINUTE BY MINUTE DETERMINATION OF UNIDIRECTIONAL AND NET WATER FLUXES IN FROG URINARY BLADDER

A REEXAMINATION OF THE TWO BARRIERS IN SERIES HYPOTHESIS

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Summary

Unidirectional and net water fluxes were simultaneously estimated in frog urinary bladder. The minute by minute tritiated water (^{3}HOH) transepithelial flux and the net volume of fluid traversing the tissue were employed. It was observed that: (1) the time course of the increase in the ^{3}HOH flux induced by antidiuretic hormone had a very similar pattern to that reported for the increase in the net movement. (2) Unstirred layers strongly affected the magnitude of the antidiuretic hormone-induced increase in ^{3}HOH fluxes while the time course of the response was almost non-affected. In non-stimulated bladders ^{3}HOH fluxes were poorly modified by medium stirring. New steady-state conditions for ^{3}HOH fluxes were established 1 min after stirring rate modifications. (3) The simultaneously determined net water flux was not affected by a modification in the unstirred layers, indicating that the variations in the measured net water fluxes are a good estimation of the changes in the mucosal border permeability. (4) The presence of an osmotic gradient during hormonal challenge (implying net water fluxes, cell swelling and dilation of the intracellular spaces) did not modify the time course of ^{3}HOH movements. These results suggest that the time course of the increase in water permeability is an intrinsic characteristic of the experimental system that could result from the addition of permeability units that increase in number during the development of the hormonal action.

Introduction

The amphibian urinary bladder, a tissue that resembles the mammalian terminal nephron in several respects, has been largely employed to study water permeability mechanisms. This epithelium has been chosen because of the modulatory effect of antidiuretic hormone on this function and because it easily allows the determination of net [1] and unidirectional water fluxes [2]. The measure of net water fluxes was highly improved with the introduction of a volumetric technique that allows the automatic registration of net water fluxes [3]. Employing this experimental approach the time course of osmotic permeability under antidiuretic hormone and other agents has been studied [4]. It is evident, however, that net water fluxes can only be measured in the presence of an osmotic gradient. In this condition the increase in water permeability induced by antidiuretic hormone is accompanied by cell swelling and dilation of the intercellular spaces. It would be of importance to know the role of these factors in the time course of the observed increase in membrane permeability. Finally, the low osmotic permeability of non-stimulated preparations makes difficult the study of permeability changes (special permeability reductions) when employing short experimental periods.

Unidirectional water movements, estimated from ^{3}HOH fluxes, can be easily measured in the absence of any osmotic gradient and even in non-stimulated preparations. Nevertheless, most of the measurements on unidirectional water permeability have been performed employing long experimental periods, making difficult the study of rapid permeability changes. This situation, together with the uncertainty arising from the unstirred layer problem, made the results obtained from comparing unidirectional and net water fluxes suspicious or at least controversial [5—7]. We think, however, that a comparative time course study would allow a reexamination of current views on water transfer measurements in epithelia.

In a previous paper it has been demonstrated that tritiated water (^{3}HOH) fluxes across toad urinary bladder achieve steady-state conditions in a few seconds [8]. On this basis we have no developed a new experimental approach to measure unidirectional water movements. This approach was employed here together with the previously mentioned volumetric technique [3]. The combined use of these two methods has made it possible to measure the minute by minute unidirectional and net water fluxes, with or without the presence of an osmotic gradient, in non-stimulated preparations or under the action of different agents modifying water permeability.

The results obtained with this experimental approach will be employed here to evaluate current views on the mechanism underlying water permeation in epithelia.

Materials and Methods

Frogs (*Rana esculenta*) originating from Central Europe, were purchased from Burgaud, 85 St. Hilaire de Rietz, Vendée, France, and kept at 20°C in running tap water at least 5 days before the experiment. The bladders were removed from pithed frogs and mounted between two lucite chambers.

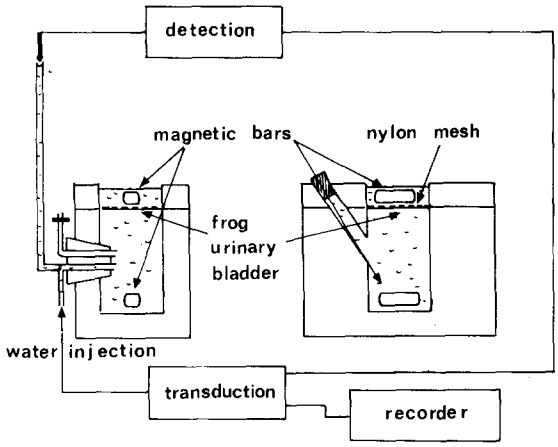

Fig. 1. Schematic representation of the chamber employed for the simultaneous determination of unidirectional and net water movements in frog urinary bladder.

Unidirectional water fluxes. The experimental set up is schematized in Fig. 1. The bladder was disposed horizontally with a nylon mesh placed on its upper side. The volume of the lower chamber was 12.5 or 6.5 ml and the volume of the upper one was 2.0 ml. Both solutions could be vigorously stirred with magnetic bars. The magnetic stirrer was below the incubation chamber. The upper magnetic bar, which was placed on the nylon mesh, very near of the superior face of the bladder (Fig. 1) followed the movement of the inferior one. In some experiments the upper magnetic bar was replaced by an electrically driven Teflon helix (no difference was detected). The bladder was mounted with the mucosal border facing the lower chamber. The lateral opening made possible the direct access to the inferior chamber. This opening was closed during net flow measurements (see below). ^{3}HOH was added to the lower chamber up to a final concentration of 10 μCi/ml. The solution in the upper chamber was then completely removed every minute and replaced with unlabelled solution. The ^{3}HOH activity of the sample was determined and the water unidirectional flux expressed in $\mu l \cdot h^{-1} \cdot cm^{-2}$ (the chamber area was 3.14 or 1.60 cm^2 in different experiments). It is important to observe that because the determination was not cumulative, the back-flux remained negligible in all cases. The specific activity in the inferior chamber was recalculated for each minute period, taking into account the previous transfer of radioactivity.

Net water fluxes. The net flux of water across the urinary bladder was measured with a technique previously described [3] which allows the every minute record of the water movement. Because the bladder was mounted with the mucosal border facing the lower chamber, net water flux proceeded from the inferior to the superior solution when the mucosal bath was made hypotonic. Water was accordingly injected into the lower chamber to maintain its volume constant. The amount of water every minute injected, and equivalent to the net flux, was recorded. Water injection was made via a polyethylene cannula, also connected to a volume detector (Fig. 1). A second tube was used to equilibrate the hydrostatic pressure before closing the lower chamber. The sensitivity of this system was of 0.05 $\mu l \cdot cm^{-2}$.

The serosal face of the bladder was bathed with a Ringer solution (Na^+ 114.5: Ca^{2+} 1.0, K^+ 5.0, Cl^- 119.0, HCO_3^- 2.5, mequiv./l in each case, pH 8.1, when bubbled with air). The mucosal side was bathed with the same saline, except in osmotic gradient experiments. In this case NaCl concentration was reduced to 5.6 mM, making this solution largely hypotonic. All experiments were performed at room temperature (about 20° C).

Results

Time course studies on water permeability. The effect of antidiuretic hormone

Fig. 2 shows a record of unidirectional (mucosa to serosa) and net water movements simultaneously determined in frog urinary bladder employing the experimental set-up described in Fig. 1. The mucosal bath was hypotonic and both solutions were maximally stirred. We can observe that the unidirectional flux increased about four times under the action of antidiuretic hormone (synthetic oxytocin, Sandoz) from 426 ± 20 to 1857 ± 92 $\mu l \cdot h^{-1} \cdot cm^{-2}$, while the net movement increased from 4.0 ± 0.6 to 190 ± 13 $\mu l \cdot h^{-1} \cdot cm^{-2}$ (about 50 times, see Table I). In some individual experiments unidirectional fluxes could reach under antidiuretic hormone up to seven times the resting value. Fluxes completely reversed to basal values upon antidiuretic hormone washout, showing that the epithelium was not damaged by vigorous stirring. Antidiuretic hormone stimulation could be repeated several times, with no significant deterioration of the system.

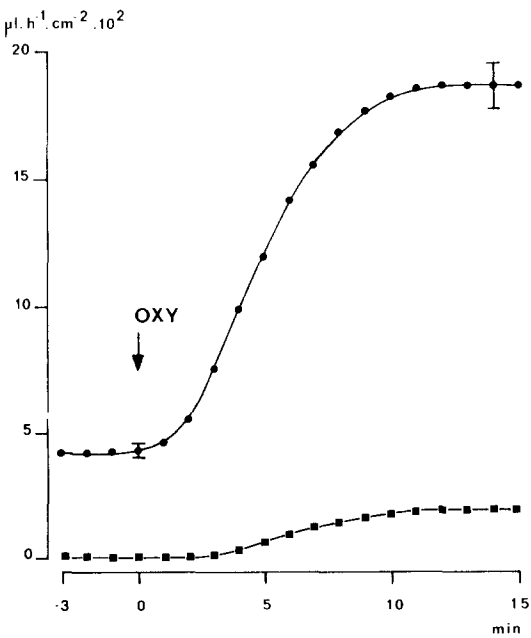

Fig. 2. ^{3}HOH unidirectional flux (upper curve) and net water movement (lower curve) simultaneously determined in frog urinary bladders in the presence of an osmotic gradient (means ± S.E. of six experiments). The drawn points cover the error in the case of the net flux). Oxytocin ($2.2 \cdot 10^{-8}$ M) (Oxy) was added at zero time.

TABLE I

UNIDIRECTIONAL AND NET FLUXES MEASURED IN DIFFERENT EXPERIMENTAL CONDITIONS

Fluxes in $\mu l \cdot h^{-1} \cdot cm^{-2}$. Mean (+S.E.) of six experiments in each case

	Control	Under oxytocin ($2.2 \cdot 10^{-8}$ M)	
		No osmotic gradient	In the presence of an osmotic gradient
Unidirectional flux			
No stirring	380 ± 18	650 ± 60	
	492 ± 52		766 ± 58
Under stirring	418 ± 22	2225 ± 87	
	426 ± 20		1857 ± 92
Net flux	4.0 ± 0.6		190 ± 13

Another interesting observation is the similarity in the time course of the response when comparing net and unidirectional water movements. In Fig. 3, water fluxes have been normalized and expressed as the percentage of the maximal variation induced by antidiuretic hormone. Again unidirectional and net water fluxes have been simultaneously determined in the same preparation (mean of six experiments). In the case of the net water movement we confirm the sigmoidal curve previously reported [4,9]. The half-time of the response to antidiuretic hormone was 6.3 ± 0.4 min ($n = 6$). For the unidirectional movement we have the same sigmoidal curve but starting slightly earlier (half-

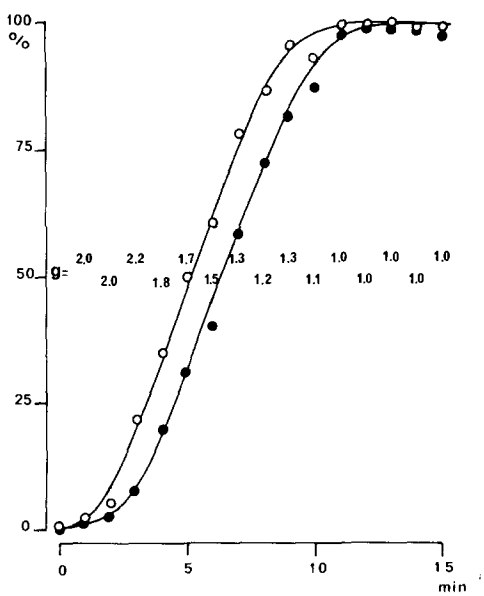

Fig. 3. Time course of evolution of the simultaneously determined ^3HOH (○) and net (●) water movements in frog urinary bladder. Fluxes are expressed as the percentage of the maximal variation induced by $2.2 \cdot 10^{-8}$ M oxytocin (mean of six experiments). The values in the middle of the figure show the every minute ratio between the percentage of increase in ^3HOH and net water fluxes (column g, Table II).

time 5.1 ± 0.4 min, $n = 6$). 6 min after antidiuretic hormone addition the distance between both curves was statistically significant (mean difference 28 ± 8%, $p < 0.01$, t-test for paired data). In fact both curves can be superposed by shifting the unidirectional one about 1 min to the right. This difference could be at least partially due to the fact that the detected net flux represents the water that has actually traversed the bladder. The fluid employed in swelling the epithelial cells and dilating intercellular spaces will not be recorded. The total net water volume traversing the tissue during the first 12 min after antidiuretic hormone stimulation was 19 ± 1.3 $\mu l \cdot cm^{-2}$. At the same time, the transfer due to the unidirectional flux was of 223 ± 20 $\mu l \cdot cm^{-2}$. The thickness of the epithelial layer can be estimated at 10 μm in frog urinary bladder [10] and, when antidiuretic hormone is added in the presence of an osmotic gradient, this epithelial layer doubles its volume [10]. This swelling represents a volume increase of about 1 $\mu l \cdot cm^{-2}$ corresponding to 5% of the total recorded net volume of water while only corresponding to 0.4% of the unidirectional movement. This will imply a delay between mucosal permeation and transepithelial net flux.

The effect of unstirred layers

It is well-known that the presence of unstirred layers strongly modifies the magnitude of unidirectional water fluxes measured employing ^{3}HOH [5—7]. The effect of bath stirring on the unidirectional and net water fluxes can be observed in Fig. 4 (both parameters were simultaneously determined and the experimental conditions were as those described in Fig. 2). When stirring was stopped there was a drastic reduction in the ^{3}HOH movement while the net flux remained almost unaffected. This clearly indicates that, under the action of antidiuretic hormone, the value of the unidirectional water movement is

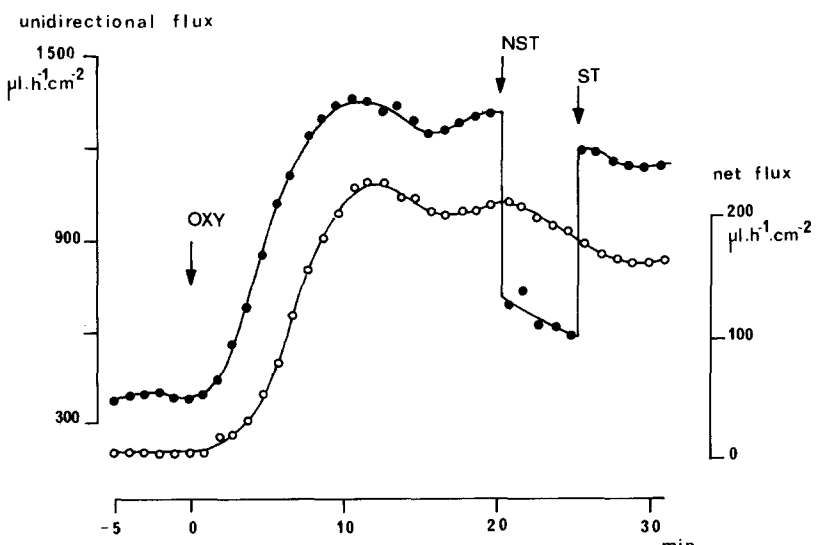

Fig. 4. Effect of medium stirring on the simultaneously determined ^{3}HOH (•) and net water fluxes (○) in frog urinary bladder in the presence of an osmotic gradient. OXY: oxytocin, $2.2 \cdot 10^{-8}$ M. The stirring was stopped (NST) once the response to the hormone was fully developed and then reinstalled (ST).

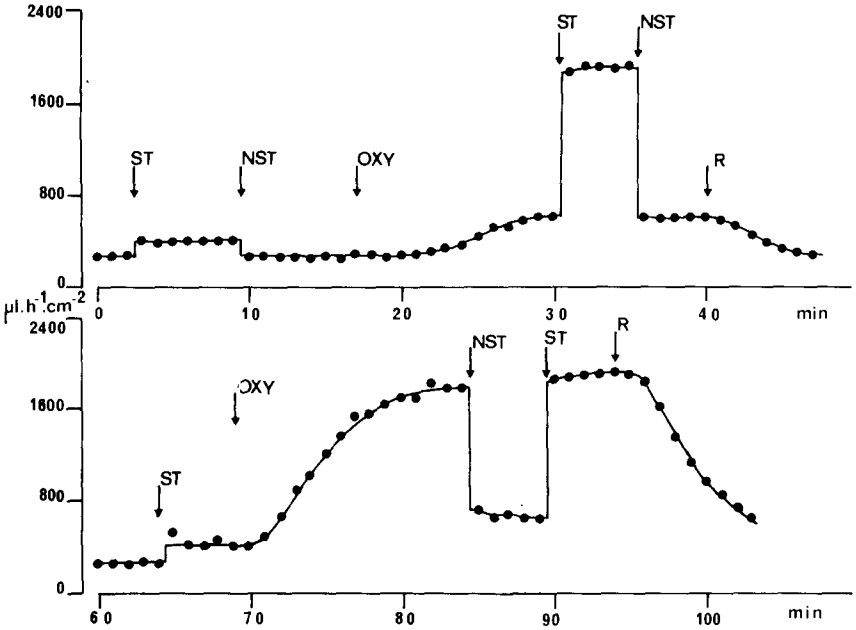

Fig. 5. Effect of medium stirring on the unidirectional ^{3}HOH movement in different situations (no osmotic gradient was applied). ST, maximal stirring rate; NST, no stirring,; OXY, oxytocin, $2.2 \cdot 10^{-8}$ M; R, oxytocin wash-out.

underestimated when employing ^{3}HOH. Another interesting observation is that the unidirectional ^{3}HOH flux reached its new steady-state value very quickly, confirming a previous observation [8].

Fig. 5 shows the effect of medium stirring on the calculated unidirectional water movement from ^{3}HOH measurements, in different conditions. No osmotic gradient was applied in this experiment. We can see that the observed flux is poorly modified by medium stirring in non-stimulated preparations. On the contrary, medium stirring introduced a strong modification in the measured flux when the hormonal action was fully developed [7,11]. It can be observed that the effect of unstirred layer modification is almost completely established as soon as 1 min after the modification of the stirring rate. The modifications induced by changing the stirring conditions are completely reversible, and can be repeated several times in the same preparation. Furthermore, they are independent of the presence of an osmotic gradient (compare Figs. 4 and 5).

We have also studied the effect of medium stirring on the time course and magnitude of the increase in ^{3}HOH permeability induced by antidiuretic hormone. The obtained results are shown in Fig. 6a, where the increase in the water flux induced by the hormone is again represented as a percentage of the maximal variation. No osmotic gradient was applied in this series. Two determinations were successively performed in the same preparation. In three experiments the permeability was first tested under maximal stirring conditions and in the three others the non-stirred condition was first examined. We have com-

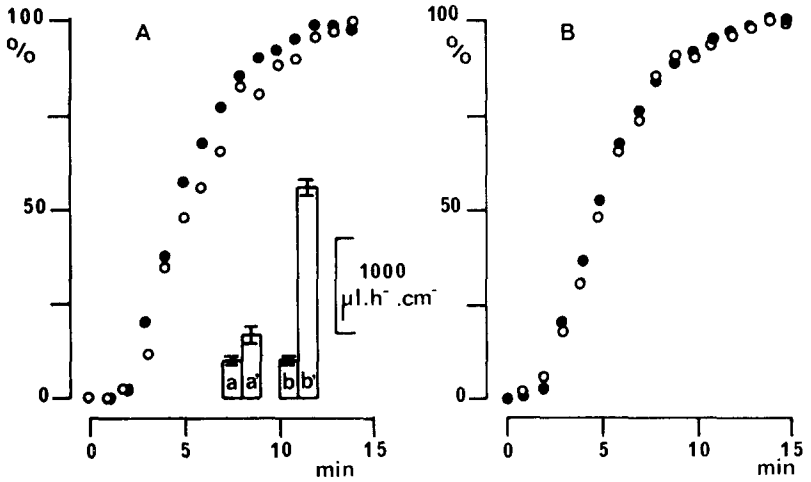

Fig. 6. (A) Effect of medium stirring on the time course evolution of the ^{3}HOH unidirectional transfer. Fluxes are expressed as the percentage of the maximal variations induced by oxytocin. No osmotic gradient was applied in this series. The experiments were performed under maximal (•) or minimal (○) stirring rates. The inset shows the magnitude of the unidirectional ^{3}HOH flux in each case: (a) no stirring, before oxytocin; (a') no stirring, after oxytocin; (b) maximal stirring, before the hormone; (b') maximal stirring, after the hormone. Mean of six experiments in each case. (B) Time course evolution of the unidirectional ^{3}HOH flux under oxytocin, $2.2 \cdot 10^{-8}$ M. Values are expressed as the percentage of the maximal variation induced by the hormone in the presence (•) or absence (○) of an osmotic gradient.

pared the time course of the response to antidiuretic hormone, with (filled dots) or without (open dots) medium stirring. Non-significant differences could be established ($t_{1/2}$ = 5.2 ± 0.3 and 5.4 ± 0.4 min, respectively). Furthermore, the sigmoidal curve is very similar to the sigmoidal curve obtained when the net flux was measured. On the other hand, upon antidiuretic hormone stimulation, the calculated unidirectional fuxes increased from 418 ± 22 to 2225 ± 87 $\mu l \cdot h^{-1} \cdot cm^{-2}$ (n = 6) under maximal stirring conditions and from 380 ± 18 to 650 ± 60 $\mu l \cdot h^{-1} \cdot cm^{-2}$ (n = 6) in non-stirred periods (see inset in Fig. 6a and Table I).

We come then to this important conclusion: unstirred layers strongly affect the calculated value for unidirectional water movement when ^{3}HOH is employed while the time course of the response to antidiuretic hormone is only poorly modified. It must be stressed here that even with maximal agitation, part of the unstirred layers are still present. If we accept that the regulatory permeability barrier is at the mucosal border of the epithelial cells, then the cytoplasm of these cells and the connective support will represent in series unstirred layers that can never be removed by medium stirring. As previously postulated by Hays and Franki [7] and observed by Parisi and Piccini [8] in uptake experiments, the actual increase in the unidirectional water movement will always exceed that measured under maximal stirring rates.

Influence of the osmotic gradient

It was also interesting to know if the presence of an osmotic gradient modified the time course of the response to antidiuretic hormone. We have observed that this is not the case. Fig. 6b compares the water unidirectional fluxes

measured in the presence of in the absence of an osmotic gradient. The observed half-times were 5.0 ± 0.4 and 5.2 ± 0.4 min (n = 6) in each case. These results indicate that the establishment of an important water gradient, implying a net water flux, cellular swelling and interspace dilation, does not interfere with the evolution of water permeability under antidiuretic hormone.

Discussion

We believe that the experimental approach described in this paper, allowing the simultaneous minute by minute determination of unidirectional and net fluxes, gives a new value to the studies on water unidirectional fluxes employing ^{3}HOH. The time course of the increase in water permeability observed under the action of antidiuretic hormone was first described employing a volumetric method [4]. This technique, measuring net water fluxes, is only operative in the presence of an osmotic gradient. On the other hand, unidirectional fluxes can be measured in the absence of any osmotic or hydrostatic gradient. The similarity between the time course of the response to the hormone in both conditions and employing independent experimental devices, clearly indicates that we are observing an intrinsic characteristic of the experimental system. This is an important conclusion because it has been postulated that the cellular hypotonicity induced by the presence of the net flux would be a negative feedback regulating mucosal permeability. Our results show that the time course evolution of water permeability under antidiuretic hormone would be independent of cellular tonicity.

Another advantage is that minute by minute unidirectional ^{3}HOH measurements allow the study of rapid water permeability variations in non-stimulated preparations, where net flux measurements require long experimental periods. We will now analyse current views on the mechanism of action of antidiuretic hormone, employing the present results.

The two barriers in series hypothesis

The existence of hydrophilic pores in the apical border of epithelia was the first explanation proposed for water translocation across these tissues [2,12, 13]. Subsequent studies on the effect of unstirred layers [5,6] showed that the observed difference between diffusional (calculated from ^{3}HOH fluxes) and osmotic permeabilities was at least partially eliminated by appropriate stirring. A two barrier in series model was then proposed [7] to explain the situation encountered in toad urinary bladder where, under the action of antidiuretic hormone, the flow increases about 50 times while the diffusional ^{3}HOH flux shows only a two-fold increase in the absence of vigorous stirring. According to this hypothesis, unidirectional fluxes would be underestimated from ^{3}HOH measurements because of tracer profile concentration in the so-called unstirred layer. The observed permeability (P_{obs}) would be related to the mucosal permeability (P_m) by the following relationship [7]:

$$1/P_{obs} = (1/P_m) + (1/P_{unst}) \quad (1)$$

where P_{unst} is the unstirred layers diffusional permeability. It is important to emphasize that P_{unst} takes into account all the non-regulatory barriers for water

diffusion present in the system, i.e. external unstirred layers, cellular cytoplasm and connective tissue. It has been previously observed that bath stirring only poorly modifies ^{3}HOH permeability in non-stimulated preparations [7,8]. We fully confirmed this observation which indicates, according to the two barriers in series model, that in non-stimulated preparations P_m is small compared to P_{unst}, so that, P_{obs} is a good estimation of the mucosal membrane permeability at rest, even in the absence of vigorous stirring.

When antidiuretic hormone is added, under the stirring conditions described in this paper, the unidirectional flux increased about four times at a moment where the net water flow was increased by 50 times (see Table I). According to the two barriers in series hypothesis this could be due to the fact that the unstirred layers, which by definition are not altered by the hormone, have become the limiting barrier. In this condition the observed diffusional permeability (P_{obs}) will be similar to the unstirred layer permeability (P_{unst}).

TABLE II

EXPERIMENTAL AND CALCULATED VALUES EMPLOYED IN FIG. 7a

Time after ADH (min)	(a) Observed net flux increase (%)	(b) Corresponding variation in mucosal permeability (P_m)	(c) Expected variation in total permeability (P_{obs})	(d) Corresponding expected ^{3}HOH flux increase (%)	(e) Observed ^{3}HOH flux increase (%)	(f) ((d)/(a))	(g) ((e)/(a))
0	0	1.00	0.80	0	0	—	—
1	1	1.49	1.08	9.6	2	9.60	2.0
2	3	2.47	1.52	24.8	6	8.26	2.0
3	10	5.90	2.38	54.5	22	7.78	2.2
4	20	10.80	2.92	73.1	35	3.69	1.8
5	31	16.19	3.21	83.1	50	2.68	1.6
6	40	20.60	3.35	87.9	61	2.19	1.5
7	58	29.42	3.52	93.8	78	1.61	1.3
8	72	36.28	3.60	96.6	87	1.34	1.2
9	81	40.69	3.65	97.9	98	1.21	1.3
10	87	43.63	3.66	98.6	92	1.13	1.0
11	98	49.02	3.70	100.0	100	1.02	1.0
12	100	50.00	3.70	100.0	100	1.00	1.0
13	98	49.02	3.70	100.0	100	1.00	1.0
14	100	50.00	3.70	100.0	100	1.00	1.0
15	97	49.02	3.70	100.0	99	1.00	1.0

The minute by minute evolution in the net water flux under antidiuretic hormone (mean of six experiments, shown in Fig. 3) was expressed as the percentage of the maximal response (column a). Because unstirred layers did not affect net water fluxes (Fig. 4) it can be accepted that this parameter reflects the actual changes in mucosal permeability. The corresponding relative permeability variations (P_m) can then be calculated (column b), known that the net water flux increased from 1 to 50, in relative units, under antidiuretic hormone (Table I). The simultaneously measured unidirectional ^{3}HOH movement (P_{obs}) only increased from 1 to 4, always in relative values (Fig. 2, Table I). Then the expected minute by minute evolution in the ^{3}HOH permeability when applying Eqn. 1 ($1/P_{obs} = 1/P_m + 1/P_{unst}$) can be determined (column c), P_{unst} was taken as being equal to 4, accepting that at the end of the response $P_{obs} = P_{unst}$. This expected evolution in P_{obs} can be expressed as the percentage of the maximal variation (column d) and compared to the experimentally observed percentual variation in ^{3}HOH fluxes (column e). Column f shows the ratio between the expected ^{3}HOH flux (calculated from eqn. 1) and the observed net flux. Column g shows the experimentally observed relationship (see Figs. 3 and 7).

Time course evolution of unidirectional and net fluxes

Two different barriers then account for unidirectional ^{3}HOH fluxes: the mucosal membrane is limiting at rest, but it is replaced by the unstirred layers when the permeability increases under the action of the hormone. Which will be the expected increase in the ^{3}HOH fluxes during the transition from one state to the other? The results presented in Figs. 3, 5 and 6 show that the time course of the increase in ^{3}HOH permeability under antidiuretic hormone is unaffected by the unstirred layers. Furthermore, the time kinetics of unidirectional and net fluxes were also similar. This will be rather unexpected if one barrier is progressively replaced by the other barrier as the limiting one, indicating that the whole membrane would be smoothly increasing its permeability. If we accept Eqn. 1, the expected difference between the time course of the increases in net and unidirectional fluxes can be estimated in the following way (see Table II): because unstirred layers did not affect net water fluxes (Fig. 4) it can be accepted that this parameter reflects the actual changes in membrane permeability (P_m). The net water flux increased from 1 to 50 in relative units under antidiuretic hormone and we know its minute by minute development. On the other hand, the simultaneously measured unidirectional ^{3}HOH movement (P_{obs}) only increased from 1 to 4, always in relative units

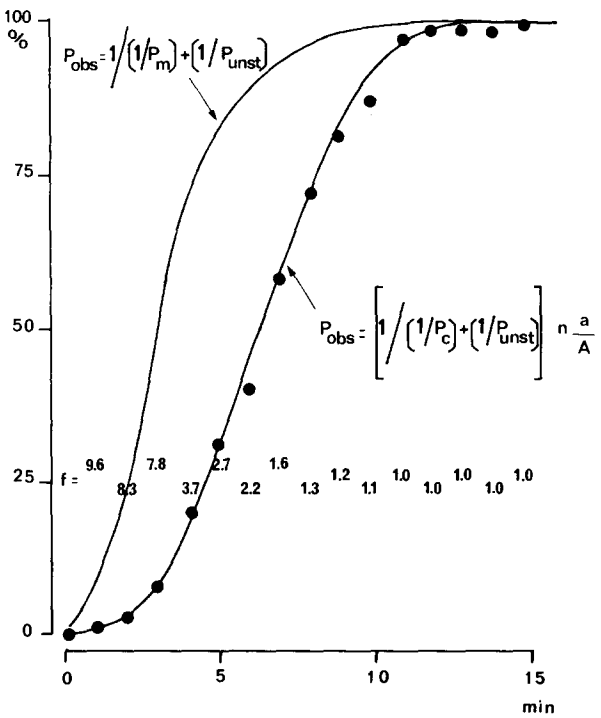

Fig. 7. The points show the observed variation in the net flux oxytocin. The left curve shows the predicted increase in ^{3}HOH flux when applying Eqn. 1: $P_{obs} = 1/((1/P_m) + (1/P_{unst}))$ (values from column d, Table II). The line on the experimental points was obtained from Eqn. 2: $P_{obs} = (1/((1/P_c) + (1/P^*_{unst}))) \; n \cdot a/A$. The values in the middle of the figure show the ratio between the expected ^{3}HOH fluxes (from Eqn. 1) and the observed net flow (column f, Table II).

(Fig. 2, Table I). The unstirred layers permeability remains constant during antidiuretic hormone action and it can be taken as equal to 4, if we accept that at the end of the response P_{obs} mainly represents the unstirred layers permeability (P_{unst}). Now the expected minute by minute increase in the ^{3}HOH permeability can be calculated applying Eqn. 1. The obtained values are shown in Table II, column c, while column d shows the expected percentual increase in the ^{3}HOH flux. Finally, the left curve in Fig. 7 shows the type of deformation expected in the time course of the ^{3}HOH flux under antidiuretic hormone, when compared with the increase of the net water flow. As previously stated, this deformation was not observed (Figs. 3 and 6). The small delay observed when the ^{3}HOH and net fluxes were simultaneously determined (Fig. 3) is probably due to the swelling of the epithelial tissue but, in any case, it does not represent the predicted modification (compare columns f and g in Table II) *.

It can be concluded that an important difference between time course of the increases in ^{3}HOH and net water fluxes is expected, if it is accepted that: (a) Eqn. 1 can describe the observed changes in unidirectional fluxes due to a modification in the mucosal permeability induced by antidiuretic hormone, and (b) the net flux is a good estimation of the real mucosal permeability.

An alternative hypothesis

From the previous paragraph the following question arises: Which would be the theoretical model compatible with the present results? Let us consider this hypothetical situation: we have a membrane with n equal channels of similar cross-section a. The total permeability (P_{tot}) will be described by the following relationship:

$$P_{tot} = P_c \cdot \frac{a}{A} \cdot n$$

where A is the total area of the membrane, and P_c the permeability of each channel. We will suppose that each channel represents two barriers in series for water diffusion, which respectives permeabilities are P_1 and P_2. If we call $a, b, c, \ldots, n$, the different parallel channels and we accept that

$$P_{1a} = P_{1b} = P_{1c} = \ldots P_{1n} = P_1$$

$$P_{2a} = P_{2b} = P_{2c} = \ldots P_{2n} = P_2$$

it follows

$$1/P_c = 1/P_1 + 1/P_2$$

and then

$$P_{tot} = \frac{1}{1/P_1 + 1/P_2} \cdot \frac{a}{A} \cdot n$$

This last relationship describes the total permeability expected from a mem-

* It is important to say that in certain circumstances unstirred layers and stagnant films can cause marked underestimations in the osmotic permeability. We have actually observed this phenomenon in special conditions not employed in this work. We think that in the experimental situation here described, as shown in other cases [6], the unstirred layers did not significantly affect the observed values.

brane having n channels of similar cross-section if each channel is composed by two barriers in series.

Which would be the physical meaning of this equation applied to our problem? We will go back to the unstirred layers and consider the situation in which a small channel (through which water can only move by restricted diffusion) is open in a membrane separating two water reservoirs. ^{3}HOH has been added to one bath and the system is in steady state. The ^{3}HOH concentration near the channel will be lower than the bulk concentration and semi-spheric surfaces, centred in the channel, will have the same ^{3}HOH activity. This concentration will increase with the distance from the channel centre and at a certain distance it will be not significantly different from the bulk concentration, even at the plane of the membrane. If we now open another channel very far from the first one, the same process will take place and we will have a second zone where the concentration gradient will be dropping to the centre of the channel. Similar concentration gradients can be described in the other side of the membrane. It must be stressed at this point that the unstirred layer is not restricted to the area in which the concentration is significantly changing. The unstirred layer is parallel to the membrane surface and probably extends far behind the point in which the concentration becomes not significantly different from the bulk concentration. When we have n sufficiently separated channels, it is evident that the observed ^{3}HOH permeability (P_{obs}) will be described by the following equation

$$P_{obs} = \frac{1}{1/P_{unst}^{*} + 1/P_c} \frac{a}{A} \cdot n \qquad (2)$$

where P_c is the permeability of each channel and P^*_{unst} is the permeability of the barrier due to the concentration gradient around each channel.

We arrive to the following conclusion: If we have a membrane containing n channels, sufficiently separated one from the other and if the number of operative channels is changing with time, the increase in ^{3}HOH permeability will be described by Eqn. 2 and the time course variation in P_{obs} will be a linear function of n (it has been assumed that each channel permeability varies almost instantaneously between zero and a maximal value). If in the same conditions we measure the net water flux, Eqn. 2 will still be valid but P_{unst} will be almost zero. In this situation P_c actually also reflects the resistance to the convective movement of water. If we accept this model for the frog urinary bladder, the time course evolution of water permeability under antidiuretic hormone would be dependent on the number of operative channels present in each moment. The addition of the hormone would induce the appearance of highly permeable areas which permeability will reach its maximum value as soon as formed. This will explain the similarity between the time course of the response to antidiuretic hormone determined employing unidirectional or net water movements. When water permeability is estimated from ^{3}HOH measurements the unstirred layers will then only modify the magnitude but not the time course of the response (Fig. 7). This last parameter will be only dependent on the specific mechanism that induce channel formation.

It is evident that the previously described ideal situation cannot be directly applied to the frog urinary bladder. Nevertheless, the observed similarity

between the time course of ^{3}HOH or net water fluxes under antidiuretic hormone will be in accordance with the existence of 'local diffusional barriers' in series with each hypothetic water channel. The total unstirred layer effect will depend, in a complex manner, on the distance between the different channels, the thickness of the unstirred layer and medium stirring.

We can conclude that the difference between the increase in unidirectional and net water fluxes under antidiuretic hormone, together with a similar time course evolution, are compatible with a system of parallel channels that become operative at different moments during the development of hormonal action. Each channel would represent a more important resistance to ^{3}HOH flux that for net water movement.

Some recent morphological data are in good agreement with the previously described hypothesis. Thus freeze-fracture studies of the mucosal border of antidiuretic hormone target epithelia have demonstrated the existence of intramembranous particles (probably proteins) that specifically and reversibly aggregate under the action of antidiuretic hormone and other agents that increase water permeability [14—16]. Moreover, the fact that no aggregates are observed in the absence of hydroosmotic agonist supports the idea that different mechanisms of water permeation are working at rest and in stimulated states. Finally, it has been recently reported that, as soon as 1 min after antidiuretic hormone addition, complete particle aggregates are already present in the membrane. The main difference between a 1 min and a 15 min stimulation seems to be the number of clusters present in the membrane [17].

In two recent papers Finkelstein [18,19] clearly analysed the possible molecular mechanism involved in the antidiuretic hormone-induced water pathway. He compared the permeability properties of water and different molecules in artificial membranes and in toad urinary bladder and concluded that the increase in water permeability induced by antidiuretic hormone cannot be explained by a modification of the lipid matrix. According to these observations water must be translocated (when antidiuretic hormone is present) across small hydrophilic channels, rather than through the lipid core. The size of these channels would be such as to exclude the establishment of any important bulk flow. Our results, showing that the presence of an osmotic gradient did not modify the time course of the response to the hormone, also suggest that the presence of a bulk flow in the water 'channel' is difficult to accept. If the water permeability increase started by antidiuretic hormone would be inducing a progressive increase in a viscous flow, in the presence of an osmotic gradient, the observed time course evolution would be different from that observed in the absence of an osmotic gradient, when only diffusional forces are present.

Further experiments are necessary to confirm the previously discussed hypothesis, but we think that the present experimental approach will be an useful tool in this way.

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