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Urea Uptake and Translocation in Toad Urinary Bladder: The Effect of Antidiuretic Hormone

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Summary. The uptake of C^{14}-urea into everted and noneverted bladder sacs was compared, over short time periods (up to 2 min), with the transepithelial urea fluxes. This method allowed the study of the time course of urea uptake and distribution, while previously this problem was only studied in steady-state conditions. When mucosal uptake was studied no accumulation of C^{14}-urea inside the tissue was observed, indicating that the mucosal border could be the limiting step. Comparative studies of urea and inulin uptake from the serosal side showed that urea equilibrated with the water epithelial cells in less than 30 sec. This accumulation suggested again that the mucosal border is an effective barrier for urea translocation. The kinetics of the increase in urea permeability induced by antidiuretic hormone was also studied and it was similar ($T^1/_2$: 4.3 min) to the kinetics of the increase in water permeability induced by the hormone ($T^1/_2$: 5.6 min). A strong parallelism was also observed between the time course of the increases in water and urea permeabilities induced by medium hypertonicity ($T^1/_2$ 25 and 26 min, respectively). The values obtained for the permeability coefficient k_{trans}), either at rest or under ADH were similar to those previously reported employing steady-state techniques (28 ± 8 and 432 ± 25 cm · sec^{-1} · 10^{-7}, respectively).

The mechanism of urea reabsorption by the renal tubule is a problem of major interest which has been widely investigated (*see* Schmidt-Nielsen, 1970). The toad urinary bladder, a tissue that resembles the mammalian renal tubule in several respects, has been largely used to study this problem. The observation that urea movement across toad bladder accelerated in the direction of net water flux (Leaf & Hays, 1962) suggested that urea translocated through true aqueous channels existing in the membrane, large enough to permit water-solute interactions. However, more recent observations demonstrated that the solvent drag effect on amide diffusion is abolished when the bathing solutions are effectively stirred

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(Hays, 1972) and that phloretin inhibits urea movements across toad urinary bladder with no inhibition of water flow or active sodium transport (Levine, Franki & Hays, 1973). From these results it was concluded that the movement of urea is independent of water flow and active sodium translocation.

Antidiuretic hormone (ADH) increases both water and urea permeability in toad urinary bladder. If a different molecular mechanism for urea and water translocation is accepted, the question arises whether both permeabilities are regulated by a common mechanism or not. To obtain further information on this point we studied the urea uptake and translocation in toad urinary bladder, as well as the time course of the response to ADH. These values are compared with the results obtained when studying water movement, specially under the action of the hormone.

In a previous work the penetration and distribution of tritiated water into the toad urinary bladder has been studied (Parisi & Piccinni, 1973), following the uptake and translocation of the tracer over short time periods. We used the same approach here when studing urea permeation. This method allowed us to study the time course of urea uptake and distribution, while previously this problem was only studied in steady-state conditions.

Materials and Methods

Experimental Approach

The toad bladder epithelium is a single layer of cells bound by two surfaces (*I* in Fig. 1). The supporting tissues (*II*) are in series with it on the serosal side, and the mucosal surface of the cells represents the luminal border. The system can be considered as a complex arrangement of barriers in series. As a working hypothesis we will assume that the only significant barrier for urea movement is the mucosal border. In this case, assuming that the serosal and mucosal bath are reservoirs, when C^{14}-urea media are placed on the luminal side one would expect that the same concentration of C^{14}-urea would be present in the bladder and in the serosal fluid. This would not be expected if the inner barriers act as limiting steps for urea movement, because in this case C^{14}-urea may accumulate within the bladder. On the other hand, if C^{14}-urea is added to the serosal bath and the mucosal border is an effective barrier for this molecule, a radiotracer accumulation inside the tissue must be expected.

These predictions were experimentally tested in the following way: first, everted bladder sacs were incubated in C^{14}-urea containing media. The total radioactivity taken up by the sacs was measured after 30, 60, and 120 sec of exposure to the tracer. This represented the C^{14} urea taken up by the tissue plus the serosal fluid contained inside the sac (Fig. 1*a*). The results obtained were compared, in parallel experiments, with the activity obtained

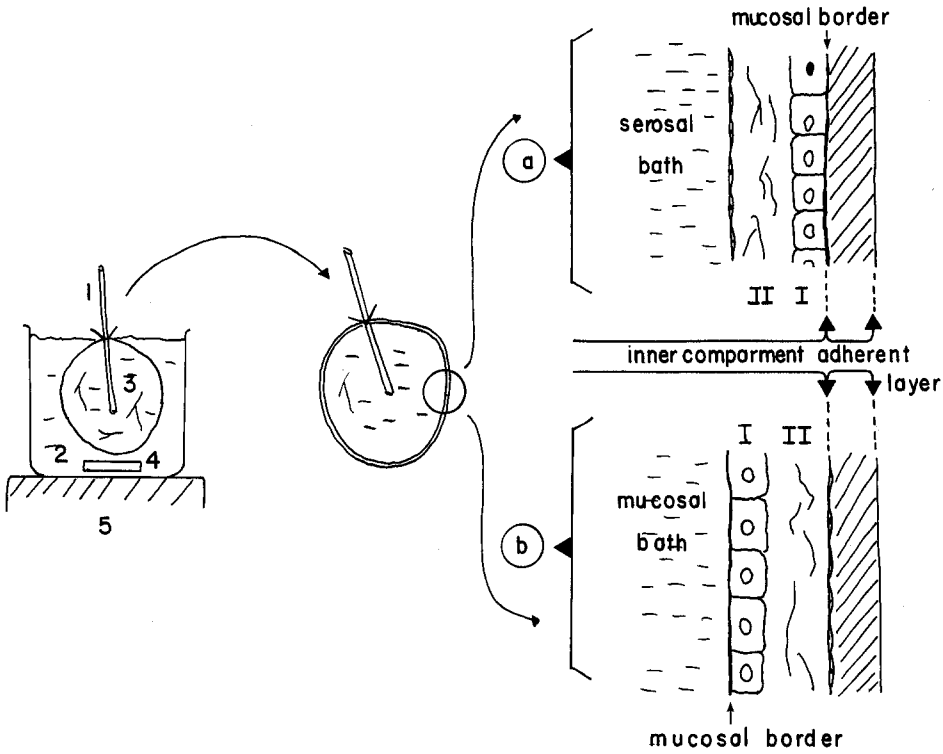

Fig. 1. Schematic representation of the experimental approach employed in uptake experiments. (*1*, *3*) Toad bladder sacs, with the polyethylene cannula inside it; (*2*) soaking solution; (*4*, *5*) magnetic bar and magnetic stirrer. After removal from the soaking solution: (*a*) Detailed schema of toad bladder when employing everted sacs; (*b*) the same, but when using non-everted bladder sacs

in samples of the serosal fluid alone. By substracting these two values, taken at corresponding times, the concentration in the tissue can be calculated.

In a second type of experiment noneverted sacs were incubated in C^{14}-urea containing media and the radioactivity take-up was measured. In this case the radiotracer accumulation in the tissue plus the mucosal bath was followed (Fig. 1 *b*).

In the following, the bladder tissue plus the fluid inside the sac will be called the "inner compartment" in both type of experiments (Fig. 1 *a* and *b*).

The influence of unstirred layers. It can be calculated that an unstirred layer of 200 Å thickness does not introduce a significant modification in the measured urea permeabilities across toad urinary bladder (Pietras & Wright, 1975). We have confirmed this prediction in our experimental conditions. The urea permeability measurements here reported, either in the presence or in the absence of vigorous serosal or mucosal stirring, were not different from those previously reported in nonwell-stirred preparations (Maffly, Hays, Lamdin & Leaf, 1960; Pietras & Wright, 1975). This is a consequence of the high free diffusion coefficient of urea in water compared to the much lower permeability across the bladder. Thus, the influence of unstirred layers on the urea fluxes measurements can be disregarded.

Experimental Procedure

Toads (*Bufo marinus*) were obtained from a commercial supplier and kept on damp earth at room temperature. The toads were pithed, their bladders removed, placed in saline (in mM: NaCl 112; KCl 5; $CaCl_2$ 1; $NaCO_3H$ 2.5; pH 8.1) and gassed with air. In some cases serosal hypertonicity was obtained by adding sucrose (up to 440 mOsm/liter) to the basic saline.

Urea Uptake by the Inner Compartment

Mucosal uptake. To perform these experiments each bladder lobe was everted and filled with about 6 ml of saline. The sac (mucosal side outward) was immersed in saline and, following a 30-min equilibration period, the net water flux was measured by weighing, following the method of Bentley (1958). They were then emptied and refilled with saline which, in some cases, contained 2×10^{-8} M oxytocin (Sintocinon, Sandoz). After 20 min the net water flux was again determined. Next the sacs were immersed for 0.5, 1 or 2 min in a saline solution containing 1 μC/ml C^{14}-urea or 1 μC/ml C^{14} inulin (Fig. 1). They were then washed for 5 sec in a nonradioactive solution, transferred to a preweighed empty beaker where the sac was cut open and the bladder bathed in the serosal fluid for 24 hr at 4 °C. 500 μl samples of the equilibrated fluid were counted for C^{14} activity in a liquid scintillation counter. The measured radioactivity represented the tracer present in the layer of fluid adhering to the mucosal surface of the cell, in the bladder tissue, and in the serosal compartment (Fig. 1*a*). The surface area was estimated from the volume of the sac, assuming a spherical shape.

Serosal uptake. To estimate serosal urea uptake noneverted bladder sacs were used. In this case the serosal side of the sac was exposed for variable times to C^{14}-urea. After sac removal the tracer will be present in the adherent layer, in the bladder tissue, or in the mucosal solution inside the sac (Fig. 1*b*).

Translocation Experiments

In this type of experiment transepithelial urea fluxes were determined. Hemibladder sacs were immersed in saline, and after checking the water net movement by weighing they were emptied and refilled with a solution containing 1 μC of C^{14}-urea per ml. The filling of the sac lasted less than 5 sec. Samples were taken from the serosal bath every min for 20 min. The measured activity was corrected for the bath volume reduction due to the sampling. The sac area was estimated as previously described.

In some cases uptake experiments were compared with transepithelial fluxes by using paired hemibladders.

In mucosal uptake experiments the mucosal bath was vigorously stirred with a magnetic bar. The serosal bath was stirred in the serosal uptake experiments and in the transepithelial (mucosa to serosa) experiments.

Results

Corrections for the Extracellular Space and Adherent Layers

When the sacs were removed from the radioactive bath the bladder mucosal or serosal surface was covered with a layer of the soaking

solution. When studying the uptake of a tracer using this method, the radioactivity present in this adherent layer, in the extracellular space, in the epithelial cells, or in the sac fluid must be taken into account (Fig. 1*a* and *b*). It has been demonstrated in previous work that inulin equilibrates in the adherent layer in less than 5 sec (Parisi, Gauna & Rivas, 1976) and that this molecule is an excellent marker of the extracellular space in toad urinary bladder (McIver & MacKnight, 1974). In the experiments described here the sacs were washed for 5 sec in a nonradioactive bath after exposure to the tracer. The purpose of this wash was to remove most of the radioactivity present at the adherent layer. The washing time period was chosen in the following way: Everted sacs were soaked in a C^{14} inulin-containing medium. After removal, they were washed for 1, 5, or 10 sec in a nonradioactive bath. It was observed that the remaining radioactivity declined exponentially and in 5 sec 90% of the radioactive inulin present in the adherent layer was removed.

After studying urea uptake by the epithelium, we made the corresponding corrections for the adherent layer and the extracellular space, according to the inulin space. The 5-sec wash was necessary to reduce the "noise" introduced by the adherent layer radioactivity.

Urea Uptake by the Inner Compartment when Using Everted Bladder Sacs

Fig. 2 shows the urea uptake by everted bladder sacs in nonstimulated preparations and under the action of ADH. The values are corrected for the adherent layer and extracellular space by substracting the inulin space. Within the 2-min period of the experiments a linear relationship between radioactivity and time was observed. From the slope of both lines the corresponding permeability coefficients were calculated (Table 1). The values obtained were similar to those previously reported for transepithelial urea permeabilities in similar conditions (Maffly *et al.*, 1960). Either in nonstimulated preparations or in the presence of ADH the experimental line extrapolates to zero radioactivity for zero time. This strongly suggests the existence of a single effective barrier for urea movements. If this is true, the barrier must be the mucosal border in both cases. This is not the case when tritiated water is moving through the same tissue (Parisi & Piccinni, 1973).

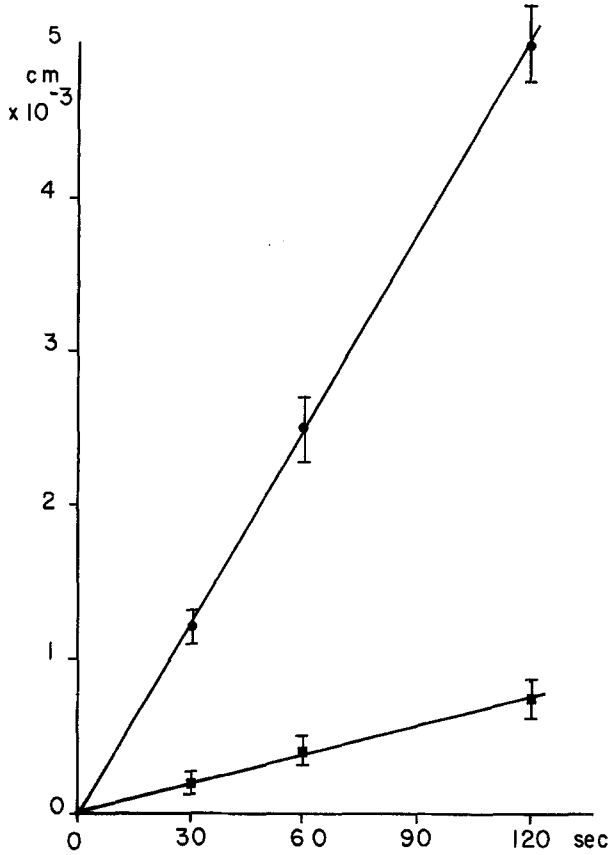

Fig. 2. C^{14}-urea uptake by everted bladder sacs. ■, control hemibladders; ●, under the action of oxytocin (n=8)

Table 1. Urea permeability in toad bladder sacs ($cm \times sec^{-1}\ 10^{-7}$)

	Mucosal uptake experiments	Serosal uptake experiments	Translocation experiments
Control	72.4±15 (n=8)	46±18 (n=8)	48±12 (n=10)
Under oxitocin 2×10^{-8} M	415±26 (n=8)	–	433±20 (n=10)

Means±SEM.

Urea Translocation across the Bladder Tissue

The results obtained in translocation experiments are shown in Fig. 3. The corresponding permeability values are presented in Table 1. These results confirm the observations made in the uptake experiments. It can be seen that in nonstimulated preparations as well under ADH

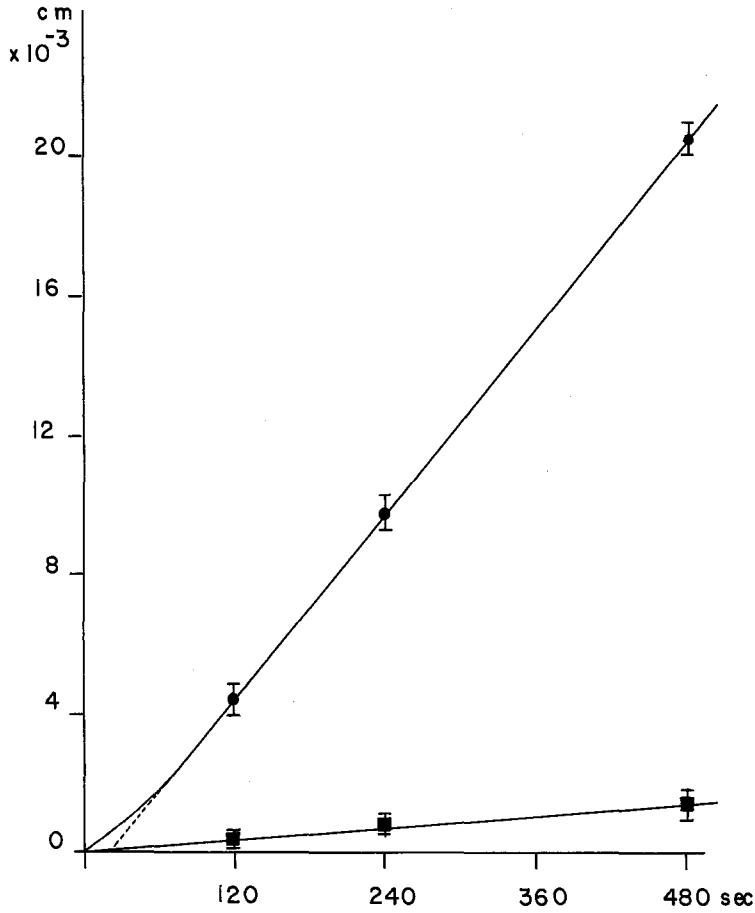

Fig. 3. C^{14}-urea appearance in the serosal compartment after exposure of the mucosal border to the tracer at zero time. ■, control; ●, under oxytocin (n=8)

there is practically no delay in the establishment of an uniform flux. The slight positive time value for zero radioactivity extrapolation shown in the graph is not statistically different from the origin.

These results also support the idea that the mucosal border is the only significant barrier for urea translocation. The permeability values were again obtained from the line slopes and they are not different from those obtained in the uptake experiments (Table 1).

Urea Uptake by the Inner Compartment when Using Noneverted Bladder Sacs

In this type of experiment the radioactive urea is present in the serosal side. After correction by substracting the inulin space, the uptake

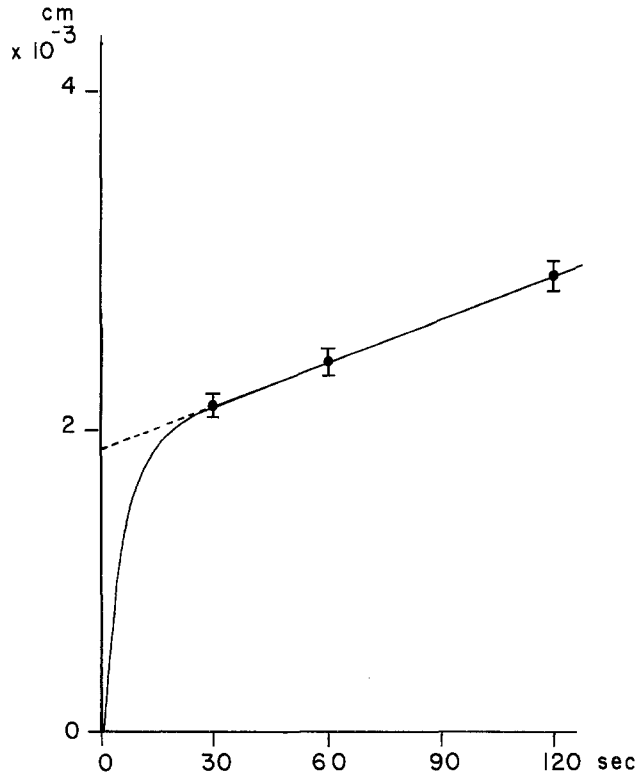

Fig. 4. C^{14}-urea uptake by noneverted bladder sacs. Control conditions. The solid line between zero time and 30 sec is tentative (n=8)

values obtained were represented against time (Fig. 4). Nonstimulated preparations were used. It can be seen that now the extrapolation to zero time discloses the presence of a fast urea compartment that reaches equilibrium in less than 30 sec. The second component is the transepithelial flux (slope: $48 \pm 10\ 10^{-7}$ cm·sec^{-1}). The first component probably represents the filling of the epithelial tissue (*see* Fig. 1*b*). The volume of the first compartment can be estimated from the ordinate at zero time. This value is equivalent to 1.92 µl · cm^2 and is an acceptable figure for the epithelial cell compartment. The immediate interpretation for these results is that the mucosal border is, as previously stated, the effective barrier for the urea movement. When the radioactivity is added to the serosal side, the mucosal barrier allows accumulation of radioactive urea within the cells.

In a few experiments we studied the urea uptake by the inner compartment in the presence of oxytocin. We observed that the volume of

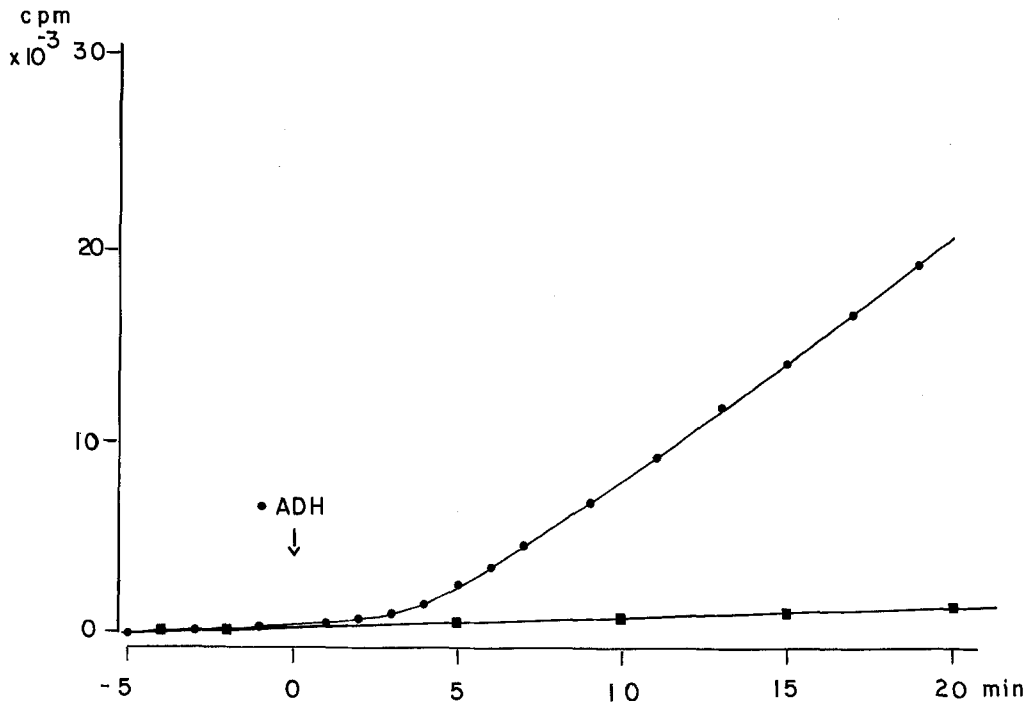

Fig. 5. Typical experiment showing the appearance of C^{14}-urea in the serosal compartment after exposure of the mucosal border to the tracer (at -5 min). ■, control hemibladder; ●, experimental hemibladder. Oxytocin (2×10^{-8}) was added to the experimental hemibladder at zero time on the serosal side

the cellular compartment was unaffected, but the slope of the transepithelial movement increased as previously observed.

Time Course of the Response to ADH: Comparison between Water and Urea Fluxes

The experimental approach employed here allowed us to study the time course of the increase in urea permeability induced by ADH. Translocation experiments were used. A typical experiment is showed in Fig. 5. From these types of experiments the minute by minute permeability increase (first derivative) was computed and the results obtained are showed in Fig. 6*a*. The mean half time of the response was 4.3 ± 0.3 min. In these experiments the effects of ADH on the water permeability was simultaneously tested (one hemibladder was used for urea measurements

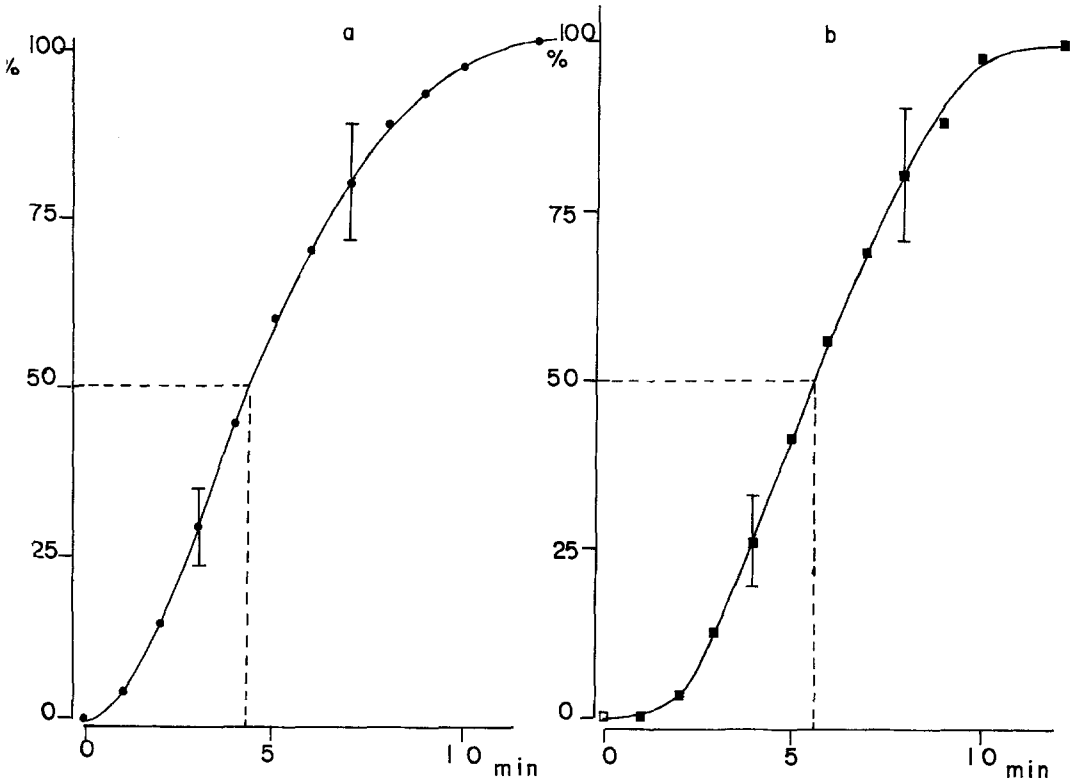

Fig. 6. Time-course of the variation in urea (*a*) and water (*b*) permeability after exposure to oxytocin (2×10^{-8} M) at zero time (n = 8)

and the other for water measurements). The net water flux was registered minute by minute and the results obtained are summarized in Fig. 6*b*. The half time of the response was, in this case, 5.6 ± 0.4 min. This value can be compared with the 7.3 ± 0.5-min figure previously reported for the water permeability increase induced by ADH in the frog urinary bladder (Ripoche, Bourguet & Parisi, 1973). When comparing the increases in urea and water permeabilities induced by the hormone, a first observation is the similarity between the patterns of both responses. Furthermore, the difference observed in the half times was not statistically significant.

The Response to Medium Hypertonicity: Comparison of the Increases in Water and Urea Permeabilities

The same approach used to study the time course of the response to ADH was used in the case of medium hypertonicity. The results obtained are shown in Fig. 7. Several conclusions can be made:

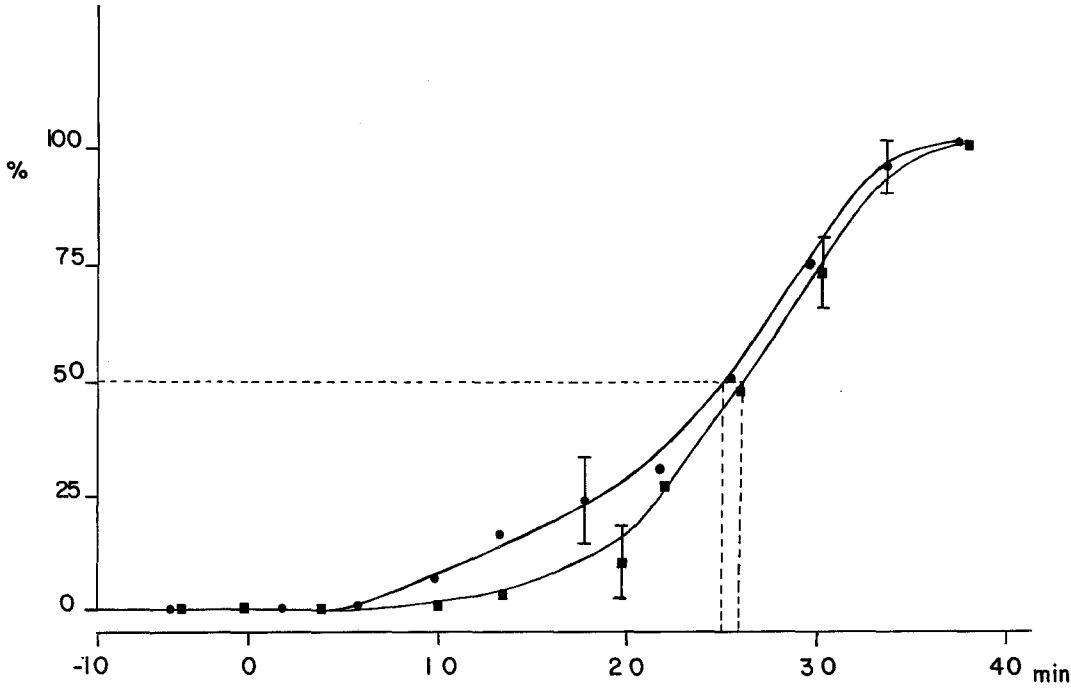

Fig. 7. Time-course of the variation in urea (●) and water (■) permeabilities after exposure to serosal hypertonicity (240 mM sucrose in the saline)

1) The time course of the responses to medium hypertonicity is slower than in the case of ADH. The mean half times for the increases in urea and water permeability were 25 and 26 min, respectively. This slow response to hypertonicity was previously reported in the case of water ($T^1/_2$ 18.9 min, Ripoche *et al.*, 1973) and is now demonstrated in the case of urea. Again, as in the case of the European frog, the scattering in the sensitivity of different bladders to hypertonicity was higher than to ADH.

2) The patterns of the responses elicited by medium hypertonicity in urea and water permeabilities are again very similar and no statistically significant differences between the experimental half times were obtained.

Discussion

The main objective of this work was to extend to the study of urea permeability an experimental approach that proved to be quite useful when working on water permeability in toad urinary bladder. The method employed allowed us to study the time course of urea uptake and distribu-

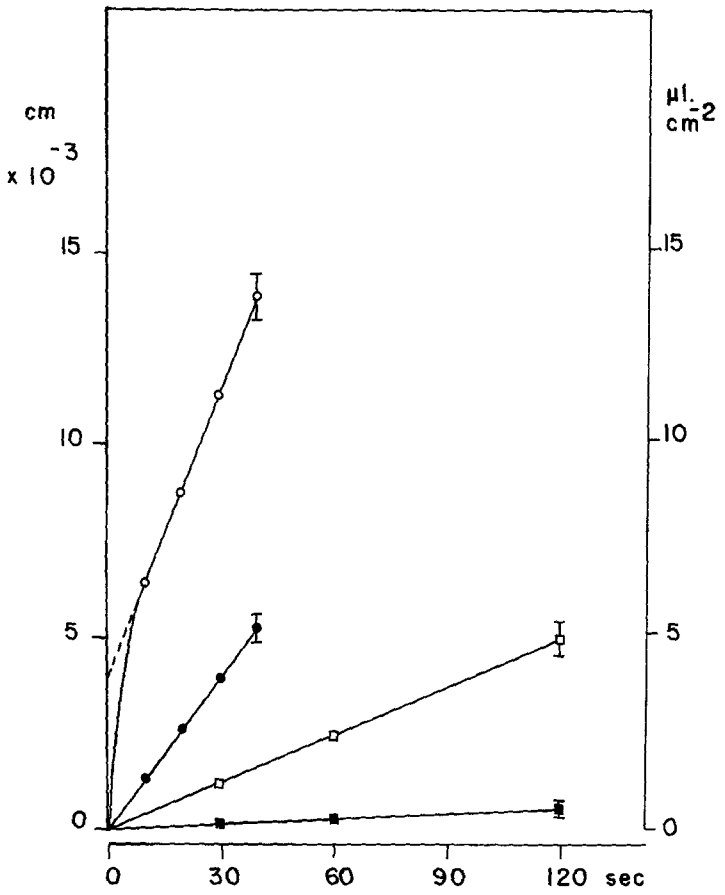

Fig. 8. Comparison between urea and water permeabilities in toad urinary bladder. ■, Urea control; □, urea under oxytocin (2×10^{-8} M); ●, water control; ○, water under oxytocin (2×10^{-8} M). The water results were taken from Parisi & Piccinni (1973)

tion and, in this case, to compare these results with those obtained when working on water uptake and distribution (Parisi & Piccinni, 1973).

The present results indicate that the mucosal border of the epithelial bladder cells is the only effective barrier for urea movement in the different conditions here studied. This conclusion is obtained from the following observations: only one compartment is apparent when the urea penetration through the mucosal border of everted bladder sacs was studied, even in the presence of ADH. In this type of experiment the rate of increase in C^{14}-urea activity in the inner compartment (the bladder tissue plus the serosal bath, *see* Fig. 1) was studied. A straight line, starting at the origin, was observed in all cases, indicating that the radioactivity

in the tissue and in the serosal bath increase at the same rate (Fig. 2). This was not the case when water penetration under ADH was studied in the same preparation (Parisi & Piccinni, 1973). Both results are compared in Fig. 8. The ordinate is expressed in cm on the left and in $\mu l \cdot cm^{-1}$ on the right. The latter represents the volume of saline present in the serosal side that might enter the bladder tissue to give account of the measured radioactivity, assuming that the tracer is translocated with all the components of this solution at the same rate. This is, of course, not true, but the figure is employed to give a clear idea of the relative permeabilities. It can be observed that in the case of water and in the presence of ADH two components are present, probably indicating the accumulation of THO inside the bladder during the first seconds after the exposure to the tracer. This implies the existence of a second effective barrier, other than the mucosal border, for water diffusion under hormonal action. This is not the case when the urea penetration into everted sacs was studied. The observation of a straight line starting at the origin, even in the presence of ADH, indicates that the bladder mucosal border is the only effective barrier for urea movements.

This conclusion is also supported by the experiments in which the urea uptake for noneverted sacs was studied. The radioactive accumulation inside the tissue when the C^{14} urea was added to the serosal bath indicates that the mucosal border is the effective barrier for urea movement. Comparing the experiments in which mucosal and serosal uptakes were employed, it is evident that the modification on the geometrical disposition of the mucosal border (*see* Fig. 1*a* and *b*) either allows or prevents the tracer accumulation inside the tissue.

Our experiments confirm the original conclusion of Maffly *et al.* (1960) based on steady-state experiments, showing that the mucosal border is the main barrier for urea movement. It must be stated, however, that the ratio between the permeabilities to water and urea does not decrease with ADH as would be expected if both molecules were penetrating the membrane by diffusion through aqueous channels.

By the method used here, the increase in permeability of the mucosal barrier can be isolated, and slopes indicate that the rates of increase are similar for urea and water. Thus, the present method is superior for representing the relative effects of ADH on permeation by urea and water (*see* Fig. 8).

The experimental approach employed here allowed us to study the time course of the permeability response to ADH. The kinetics of the

water permeability increase induced by the hormone has been previously studied by Bourguet (1967). These results have been reproduced here and compared to the pattern of the evolution in the urea permeability increase. A strong parallelism between both responses have been observed (Fig. 6*a* and *b*).

It has been previously reported that serosal hypertonicity increases water and urea permeabilities by a mechanism strongly related to cyclic-AMP levels (Ripoche, Bourguet & Parisi, 1972). By many of its characteristics the hydrosmotic response to hypertonic media compares to the physiological hydrosmotic response of the bladder to ADH. However, the response is significantly slower. We have also reproduced this difference: the increase in water permeability induced by serosal hypertonicity is slow, as compared with the action of ADH. But the interesting thing is that the increase in urea permeability induced by hypertonicity is also slower than under ADH, again a parallelism with the increase in water translocation.

As stated in the introduction, it has been recently proposed that water and urea translocate by independent pathways (Levine *et al.*, 1973). We have observed here a strong parallelism between water and urea permeabilities affected by ADH. These results are compatible with the idea of a common receptor system for both species and a post-cyclic AMP bifurcation in the ADH-dependent mechanism. However, these results are also compatible with two completely independent systems having similar reactivities to ADH and serosal hypertonicity.

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