Nature of the Water Permeability Increase Induced by Antidiuretic Hormone (ADH) in Toad Urinary Bladder and Related Tissues

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ABSTRACT In artificial lipid bilayer membranes, the ratio of the water permeability coefficient ($P_e$ (water)) to the permeability coefficient of an arbitrary nonelectrolyte such as n-butyramide ($P_e$ (n-butyramide)) remains relatively constant with changes in lipid composition and temperature, even though the individual $P_e$'s increase more than 100-fold. I propose that this is a general rule that also holds for the lipid bilayers of cells and tissues, and that therefore if $P_e$ (water)/$P_e$ (solute) greatly exceeds the value found for artificial lipid bilayers (where "solute" is a molecule, such as 1,6 hexanediol or n-butyramide, that crosses the cell membrane by a solubility-diffusion mechanism without the aid of a special transporting system), then water crosses the cell membrane via aqueous pores. Applying this criterion to the toad urinary bladder, we find that even in the unstimulated bladder, water probably crosses the luminal membrane primarily through small aqueous pores, and that this is almost certainly the case after antidiuretic hormone (ADH) stimulation. I suggest that ADH stimulation ultimately leads either to formation (or enlargement) of pores, by the rearrangement of preexisting subunits, or to an unplugging of these pores.

INTRODUCTION

Three criteria are traditionally cited as evidence for aqueous pores in cell membranes or tissues: (a) ratios of osmotic permeability coefficient ($P_f$) to tracer permeability coefficient of water ($P_d$(H2O)) significantly greater than 1, (b) solvent drag of solutes accompanying osmotic flow, and (c) graded permeability to small molecules (molecular sieving). Unstirred layers make it impossible to establish the true existence of the first criterion in almost all cases, with the possible exception of the red cell (Dainty, 1963), and also compromise the second criterion (Hays, 1972). This leaves molecular sieving, which can convincingly establish the existence of pores in some cases (e.g. Beggiatoa [Ruhland and Hoffmann, 1925]), but not if the pores admit only water (or water and very small solutes such as acetamide and urea). The water and nonelectrolyte permeability of lipid bilayers (Finkelstein, 1976), however, suggest a new criterion for deciding the path of water movement, which I shall apply to the action of antidiuretic hormone (ADH).

Until recently, most physiologists believed that ADH caused an increase in the
size of aqueous pores in the outer surface of frog skin (Koefoed-Johnsen and Ussing, 1953) and the luminal (or mucosal) surface of toad urinary bladder (Hays and Leaf, 1962). That belief rested on observed differences between $P_t$ and $P_d$(H$_2$O) as well as solvent drag effects (Koefoed-Johnsen and Ussing, 1953; Andersen and Ussing, 1957; Hays and Leaf, 1962, Leaf and Hays, 1962). Since those observations probably result from artifacts of unstirred layers (Hays, 1972), however, they do not support the theory that water crosses those tissues through aqueous pores. In fact, there is an emerging view that water traverses the luminal membrane of bladder and collecting tubules by a solubility-diffusion mechanism, and that ADH increases water permeability by increasing membrane fluidity (Schafer et al., 1974; Pietras and Wright, 1975). The results of the previous paper (Finkelstein, 1976) bear directly on this question, and, as we shall see, argue strongly for water crossing ADH-stimulated tissues through aqueous pores.\(^1\)

**Theory**

The pertinent observation from bilayers is that $P_d$(water)/$P_d$(solute) remains relatively constant with changes in lipid composition and temperature, even though the $P_d$'s change by as much as a 100-fold (Finkelstein, 1976). I propose that this is a general rule that holds for all lipid bilayers. Therefore, if water traverses the lipid bilayer of a cell or tissue primarily by a solubility-diffusion mechanism, $P_d$(water)/$P_d$(solute) should approximate the value found in artificial bilayers (for those solutes [such as 1,6 hexanediol, 1,4 butanediol, isobutyramide, and n-butyramide] that permeate cell membranes without aid of special transporting systems [see Table I]). Conversely, if $P_d$(water)/$P_d$(solute) greatly exceeds the corresponding value in the first three rows of Table I, strong evidence exists that water crosses the membrane by an alternative mechanism (e.g. via pores).

**Application of the Theory to the Action of ADH on the Toad Urinary Bladder**

**Reasons for Believing that Water Goes through Pores in the Toad Bladder**

Consider the n-butyramide and water permeabilities of the sphingomyelin:cholesterol (SC) membrane at 14.5°C (Table II, column 3) compared to those of unstimulated toad bladder mucosal membrane (the limiting permeability barrier) (Table II, column 4). The n-butyramide permeabilities are essentially the same, thus indicating that the two bilayers are of equal tightness. On the

\(^1\) It is generally agreed that the chief barrier to water movement is the luminal (mucosal) surface of the toad urinary bladder and mammalian collecting tubules, and that ADH acts by increasing its permeability (Handler and Orloff, 1973). (In the fully stimulated bladder, the water permeability of the luminal surface may be so large that other series barriers [e.g. the serosal membrane of the epithelial cells] now offer significant resistance to water movement and hence help determine the value of $P_r$.) There remains the issue, however, of whether water moves across the luminal plasma membrane of the epithelial cells or through the "tight" junctions between those cells. All evidence favors the former (Givan and DiBona, 1974); in fact there is good evidence that ADH affects the water permeability of the luminal plasma membrane of the granular cells (DiBona et al., 1969). We shall therefore assume that the permeability barrier is the luminal plasma membrane of the single layer of epithelial cells lining the lumen of the bladder and collecting tubules.
other hand, $P_d$ (water) of unstimulated toad bladder is six times larger than that of SC bilayer at 14.5°C, suggesting that even in the absence of ADH, water moves through a path separate from that of butyramide. After maximal ADH stimulation of the bladder, $P_d$(H$_2$O) increases more than 15-fold to over $2 \times 10^{-3}$ cm/s

| Membrane | $P_d$(H$_2$O)/$P_d$(n-butyramide) | $P_d$(H$_2$O)/$P_d$(iso-butyramide) | $P_d$(H$_2$O)/$P_d$(1,4 butanediol) | $P_d$(H$_2$O)/$P_d$(1,6 hexanediol) |
|----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| SC, 14.5°C | 4.1 | - | - | - |
| SC, 25°C | 2.8 | 6.9 | 27 | 1.8 |
| LC, 25°C | 1.9 | 2.9 | 29 | 2.6 |
| Unstimulated bladder | 24 | 81 | 81 | 18 |
| Stimulated bladder | 600 | 1,400 | 1,900 | 180 |

The figures for sphingomyelin:cholesterol membranes (SC) and lecithin:cholesterol membranes (LC) are calculated from the data in Table I of the preceding paper (Finkelstein, 1976). The figures for unstimulated and stimulated bladder are calculated from the data in Table II of this paper, where we have assumed that $P_d = P_t$ and that $P_t = 5 \times 10^{-4}$ cm/s (Hays, 1972).

**TABLE II**

Comparison of the values of the permeability coefficients ($P_d$'s) of water and nonelectrolytes for sphingomyelin:cholesterol (SC) membranes to those for toad urinary bladder.

| Molecule | SC, 25°C | SC, 14.5°C | Bladder, (unstimulated) | Bladder, (stimulated) | $P_d$(SC, 25°C) | $P_d$(SC, 25°C) |
|----------|----------|----------|---------------------|---------------------|----------------|----------------|
| 1,6 Hexanediol | 450 | 72 | 108 | 0.16 | 0.24 |
| n-Butyramide | 288 | 51 | 54 | 86 | 0.19 | 0.30 |
| Iso-butyramide | 118 | 16 | 35 | 9.14 | 0.30 |
| 1,4 Butanediol | 30 | 16 | 27 | 0.53 | 0.90 |
| H$_2$O | 810 | 210 | 1,500 | >13,000 | 1.6 | >16 |
| Acetamide | 21 | 16 | 72 | 0.76 | 3.4 |
| Urea | <6.1 | 14 | 49 | >2.3 | >8 |

* Finkelstein, 1976 (SC stands for sphingomyelin:cholesterol bilayer).
† Wright and Pietras (1974).
§ Pietras and Wright (1975).
|| 6.1 × 10$^{-3}$ cm/s is the value for $P_d$(urea) on lecithin:cholesterol bilayers (Finkelstein, 1976).

(Hays, 1972). The exact value is immeasurable because of unstirred layer problems, but assuming for the moment that water traverses the bilayer by a solubility-diffusion mechanism, $P_d$(H$_2$O) = $P_t$ = 5 × 10$^{-3}$ cm/s (Hays, 1972). On the other hand, $P_d$ (n-butyramide) increases only by 60% to 86 × 10$^{-7}$ cm/s (Pietras and Wright, 1975). Thus, the ADH-treated bladder has an n-butyramide permeability less than twice that of the SC bilayer at 14.5°C, but a water permeability
more than 200 times that of the same bilayer. In other words, $P_d(H_2O)/P_d(n$-butyramide) in the ADH-treated bladder is more than 100-fold greater than in the SC bilayer at 14.5°C. I believe that this is compelling evidence that water does not cross the mucosal surface of the ADH-stimulated bladder by a solubility-diffusion mechanism, but instead crosses through aqueous pores.

FURTHER REASONS FOR REJECTING A SOLUBILITY-DIFFUSION MECHANISM FOR WATER TRANSPORT IN THE TOAD BLADDER Pietras and Wright (1974) found that the permeabilities of molecules that presumably cross the mucosal bilayer by a solubility-diffusion mechanism (e.g. nicotinamide, butyramide, caffeine, and hexanediol) increase modestly (at most twofold) upon ADH stimulation, and therefore concluded (rightly, I believe) that ADH stimulation increases the fluidity of that bilayer. They further suggested, however, that the much larger ADH-induced increases in water, urea, and acetamide permeabilities (molecules which they call "hydrophilic" as distinct from the others which they call "lipophilic") result from this same mechanism. They state: "It is now recognized that the partition of solutes into biological and artificial membranes probably varies with position in the membrane; lipophilic solutes are expected to be partitioned mainly into the hydrocarbon core of the membrane, whereas hydrophilic solutes are probably located nearer the polar head groups of the lipids. [Reference to Diamond and Katz, 1974.] Consequently, changes in fluidity of a membrane could produce quantitatively different effects on lipophilic and hydrophilic solutes [my italics]. If, for example, ADH caused a relatively larger increase in the fluidity at the periphery than in the core of the bladder membranes, there should be a greater increase in the permeability of hydrophilic solutes than lipophilic solutes."

This statement actually prompted the present study (Finkelstein, 1976). On theoretical grounds it is not reasonable. The permeability barrier is the hydrocarbon core of the membrane; therefore, only partitioning into this region is relevant, regardless of whether a molecule is hydrophilic or lipophilic. But theory aside, I wanted to see if fluidity changes in lipid bilayers could produce huge increases in water permeability with only modest increases in permeability for larger, more lipophilic molecules (e.g. butyramide).

2 It is also more than 100-fold greater than in the SC bilayer at 25° or the LC bilayer at 25° (Table I, column 2). I have focused on the SC bilayer at 14.5°, because its permeability to n-butyramide is so close to that of the bladder that a comparison can be made of systems of equal "tightness." Even at 25°C, however, $P_d$ values on SC membranes for 1,6 hexanediol, isobutyramide, and n-butyramide are only about six times larger than those on unstimulated toad bladder (Table II, column 6). ($P_d$(1,4 butanediol) is only 1.9 times larger. This may result from a spuriously high value of $P_d$(1,4 butanediol) on the unstimulated [and also on the stimulated] bladder, because of contamination of [1,4-14C]butanediol with a few percent of a relatively lipophilic material [Finkelstein, 1976].) The SC bilayer at 25° is thus of comparable tightness to the bilayer of the bladder's mucosal membrane. Even the discrimination between isobutyramide and n-butyramide by the SC bilayer is comparable to that by the bladder. On the other hand, $P_d$(acetamide) of SC bilayer at 25° is about equal to that of the unstimulated bladder and $P_d$(H2O) of SC bilayer at 25° is about one-half that of the bladder (Table II, column 6). In other words, the unstimulated toad bladder is 5-10 times more permeable to acetamide and water than might be expected from its permeability to the larger solutes. It is also much more permeable to urea, even more so than is the "looser" LC bilayer. Thus, even in the non-ADH-treated bladder, these small molecules (H2O, acetamide, and urea) may permeate primarily via a separate pathway from that of the larger molecules.
I found no such effect (Finkelstein, 1976); on the contrary, there was a small trend in the opposite direction. Whether fluidity was increased by removing cholesterol (going from LC to L), changing the phospholipid (going from SC to LC), or raising the temperature (going from SC [14.5°] to SC [25°]), the increase in $P_d$ for the lipophilic solutes ($n$-butyramide, isobutyramide, 1,4 butanediol, and 1,6 hexanediol) and indeed for the other hydrophilic solutes (formamide, acetamide, and urea) was (within experimental error) either equal to or slightly greater than the increase in $P_d$(water), in striking contrast to the action of ADH (see especially Table I). Even the increase in permeability produced by phloretin (probably by a fluidity change) was generally smaller (and never larger) for water than for the companion solute tested. Thus, neither physical theory nor experiments with lipid bilayers offer the slightest support to the notion that the large ADH-induced increases in the water permeability of toad bladder result from a greater solubility and/or diffusion constant of water in the mucosal bilayer. Consequently, I believe that ADH stimulation ultimately leads to the creation of aqueous pores.

**Nature of ADH-Induced Pores**

**The Size of the Pores** The ADH-induced pores must be very small (~2-Å radius), admitting H$_2$O and possibly acetamide and urea. In the cortical collecting tubules of the kidney, ADH increases only water permeability (Grantham and Burg, 1966); apparently these pores, at least, are too small to accommodate acetamide and urea. (Lest one feel that a pore through which only water can pass strains the definition of a pore, gramicidin A, an unambiguous pore-former in lipid bilayers [Hladky and Haydon, 1972], creates pores that are permeable to water but not to urea [Finkelstein, 1973].) Since, $P_d/P_d$(H$_2$O) in such pores is probably not much greater than 1, this criterion for pores is not very useful, even if unstirred layers were not a problem.

**How Might ADH Stimulation Lead to Pore Formation?** The rapidity of the ADH response makes it unlikely that de novo synthesis of pore formers (probably proteins) occurs. I propose that the pores (or their subunits) preexist in the luminal membrane, but most of them are not patent. They might be

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3 These were double-label experiments in which $P_a$s for THO and a $^{14}$C-solute were first measured in the absence of phloretin, phloretin then added to the same membrane, and the subsequent $P_a$s determined.

4 The ability of phloretin to inhibit ADH-induced urea and acetamide permeability without affecting water permeability (Levine et al., 1973) suggests that the urea and acetamide permeability pathway is separate from that of water. There is, however, an alternative possibility. Since $P_d$(H$_2$O) in the ADH-stimulated bladder is several hundred-fold larger than $P_d$(urea) (Hays, 1972), the aqueous pore may just barely admit urea. Therefore, if phloretin either slightly reduces the pore radius or slightly obstructs the pore entrance, urea permeability would decline almost to zero without water permeability being affected. Interestingly, there is a 50% reduction in water permeability at maximal phloretin concentrations (Levine et al., 1973). The recent finding by Levine et al. (1976), however, that at certain concentrations some general anesthetics inhibit water permeability without affecting urea permeability, appears to favor independent pathways for urea and water transport.

5 This can be argued theoretically (Manning, 1975) and from the observation that $P_d/P_d$(H$_2$O) is only 5 for nystatin and amphotericin B pores, which admit nonelectrolytes up to the size of glucose (Holz and Finkelstein, 1970).
"plugged" by some molecule, or the subunits forming the pore may be so arranged that the opening is either nonexistent, or too small to admit water. ADH then leads (via cyclic AMP, etc.*) to unplugging of the pore, or rearrangement of the subunits to form an opening (or a larger opening). Perhaps the fluidity change observed by Pietras and Wright (1974) allows the subunits to form a more open configuration, or allows the junction of "half pores" (located in the inner and outer leaflets of the luminal bilayer) to form a complete, water-permeable pore (Chevalier et al., 1976).

This work was supported by NSF grant no. BMS 74-01139.

Received for publication 5 March 1976.

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* The prevailing view is that ADH acts indirectly by stimulating an adenylcyclase, leading to increased intracellular levels of 3',5' cyclic AMP and ultimately, through God knows how many steps, to alteration of luminal membrane permeability (Handler and Orloff, 1973). The suggestions that ADH acts directly on the luminal membrane (Graziani and Livne, 1971; Pietras and Wright, 1974) are not persuasive.
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