J. Membrane Biol. 22, 107–123 (1975)

The Membrane Action of Antidiuretic Hormone (ADH) on Toad Urinary Bladder

Richard J. Pietras and Ernest M. Wright

Department of Physiology, University of California Medical Center, Los Angeles, California 90024

Received 18 November 1974; revised 6 February 1975

Summary. Radioactive tracer and electrical techniques were used to study the transport of nonelectrolytes and sodium, respectively, across toad urinary bladders in the presence and absence of ADH. The permeability of lipophilic molecules was roughly proportional to bulk phase oil/water partition coefficients both in the presence and absence of hormone; i.e., ADH elicited a general nonselective increase in the permeation of all nine solutes tested. The branched nonelectrolyte, isobutyramide, was less permeable than its straight-chain isomer, *n*-butyramide, in control tissues. ADH reduced the discrimination between these structural isomers. Hydrophilic solutes permeated more rapidly than expected. In the presence of hormone, there was no change in the permeation of large hydrophilic solutes considered to move via an extracellular pathway, but there was a marked increase in the permeability of water and other small hydrophilic solutes. Collectively, these results suggest that ADH acts to increase the motional freedom or fluidity of lipids in the cell membrane which is considered to be the preferred pathway for the permeation of lipophilic and small hydrophilic molecules. At concentrations of cAMP and ADH which elicit equivalent increments in the short-circuit current, the effects of these agents on nonelectrolyte transport and membrane electrical conductance are divergent. Such observations suggest that some membrane effects of ADH may not be directly dependent upon cAMP. ADH in the mucosal solution increased the permeability of the toad bladder when the surface charge on the outer surface of the apical membrane was screened with the polyvalent cation, La^{3+}. These experiments emphasize that interaction of ADH with membranes of toad urinary bladder may account for at least some effects of this hormone.

It is well known that antidiuretic hormone (ADH) increases the permeability of the toad urinary bladder and a variety of other tissues to water and small polar nonelectrolytes (*cf.* Hays, 1972; Handler & Orloff, 1973). Since there is still much uncertainty about the mechanisms involved, we have undertaken a detailed study of the effects of ADH on the permeability of the toad urinary bladder to nonelectrolytes. Permeability measurements offer unique insights into the nature of biological membranes (*cf.* Wartio-

varra & Collander, 1960; Diamond & Wright, 1969), and the action of hormones which modify membrane permeability.

We have previously characterized nonelectrolyte permeation across the toad urinary bladder in the absence of hormones (Wright & Pietras, 1974). The permeation of lipophilic solutes across this epithelium is controlled by *i*) the solute partition coefficients (K_{oil}) which are directly proportional to permeability coefficients (P), *ii*) the hydrophobic nature of the plasma membranes, and *iii*) the "fluidity" of the membrane hydrocarbons. The permeation of hydrophilic solutes is controlled by *i*) the size (molecular volume, MV) of the solute, *ii*) the absence or presence of special mechanisms in the plasma membranes, e.g. pores, carriers or simply the configuration of the membrane lipids, and *iii*) the presence or absence of extracellular shunts. The unstirred layers and the lateral intercellular spaces are also important resistance barriers for rapidly permeating solutes.

In this study of ADH action on the toad bladder, we have sought to clarify whether or not the hormone modifies *i*) the composition and/or configuration of the membrane lipids, *ii*) the special pathways for the permeation of small polar solutes, or *iii*) the extracellular pathways for the large ($MV > 80$ cc/mole) hydrophilic solutes. Furthermore, since it is supposed that 3′,5′-cyclic adenosine monophosphate (cAMP) is an intracellular mediator of the ADH response (*cf.* Handler & Orloff, 1973), we have set out to compare and contrast the effects of ADH and cAMP on solute permeation across the bladder. We find that ADH produces a general, nonselective increase in the permeation of lipophilic solutes and a decrease in the discrimination between straight and branched-chain isomers which we interpret as an increase in the fluidity of the plasma membranes. There was no change in the permeation of the large hydrophilic solutes but there was a marked increase in the permeability of water and the small hydrophilic solutes. This increase in permeability may be related to the increase in membrane fluidity. The gross transport effects of cAMP and ADH are divergent, and this, together with the observations of others, suggests that some effects of ADH on membrane transport may not be directly dependent upon alterations in cellular biochemistry via adenyl cyclase. Preliminary accounts of some of these observations have been presented elsewhere (Pietras & Wright, 1974*a*, *b*).

Materials and Methods

The approach to this problem was similar to that used previously to study nonelectrolyte permeation across the rabbit gallbladder, toad urinary bladder and choroid plexus (Smulders & Wright, 1971; Wright & Pietras, 1974). Urinary bladders were

removed from toads (*Bufo marinus*) and were mounted as flat sheets between two lucite chambers. The area of the window between the chambers was 1.3 cm^2, and each chamber held 16 ml of saline. The composition of the saline was (in mM): 104.5 NaCl, 2 KCl, 1 $CaCl_2$, and 1 $MgSO_4$ buffered at pH 7.4 with 2.125 mM Na_2HPO_4 and 0.37 mM NaH_2PO_4. The saline in each chamber was stirred vigorously with bar magnets and was gassed with 100% oxygen. All experiments were carried out at 22–24 °C (room temperature).

Nonelectrolyte permeability coefficients were determined by radioactive tracer techniques. A tracer quantity of the radioactive (^{14}C or ^{3}H) nonelectrolyte was added to the mucosal solution, and the rate of appearance of the isotope on the serosal side was determined by withdrawing aliquots of the serosal solution at regular intervals of time. After each aliquot was withdrawn the volume of the serosal solution was maintained constant by the addition of "cold" saline. Samples were also taken from the mucosal solutions throughout the experiments. All radioactive samples were assayed by conventional liquid scintillation counting techniques where each sample was counted to a standard error of 1% or less. Permeability coefficients were expressed as cm/sec. Although most flux experiments were measured in the mucosal-to-serosal direction, a few were carried out in the reverse direction. There was no detectable difference between the two unidirectional fluxes.

The final concentration of the nonelectrolytes in the saline ranged from 30 to 50 μM; i.e., the amount added as the radioactive tracer to 16 cc of solution. However, in a few experiments, the nonelectrolyte concentration was increased to 5 mM in both the mucosal and serosal solutions. The permeability coefficients obtained in the presence and absence of "cold" solute were not significantly different.

Radioactive isotopes were purchased from International Chemical and Nuclear Company, Irvine, California, Amersham/Searle Corporation, Arlington Heights, Illinois, New England Nuclear, Boston, Massachusetts, American Radiochemical Corporation, Sanford, Florida, Dhom Products Ltd., Los Angeles, California and Cal Atomic, San Diego, California.

The unidirectional tracer fluxes usually reached a steady state within one hour after addition of the isotope, and remained constant for at least 6 hr. Antidiuretic hormone (ADH, pitressin or arginine vasopressin, obtained from Parke-Davis Company, Detroit, Michigan) or 3′,5′-cyclic adenosinemonophosphate (cAMP, from Sigma Chemical Co., St. Louis, Missouri) was added to the serosal solution 180 min after the start of the experiment. The final concentrations of ADH and cAMP were 130 mU/ml and 10 mM, respectively; i.e., concentrations known to produce maximal responses in the bladder. The permeability coefficients quoted in the text for the untreated bladder were generally derived from the fluxes during the 60-min period prior to the addition of the hormone (or cAMP). The permeabilities in the presence of the hormone (or cAMP) were taken from the fluxes measured during the 60 min following addition of the hormone.

During all the experiments reported here, we monitored the spontaneous transmural potential difference (p.d.) and conductance as described previously (Wright, Barry & Diamond, 1971). The resistance of the urinary bladders used in this study ranged from 1400 to 6500 ohms cm^2 with a mean value of 2600 ± 70 (150) ohms cm^2. All bladders having a resistance of less than 1400 ohms were discarded.

Diffusion coefficients for the nonelectrolytes were obtained either from the literature or from the Stokes-Einstein relation ($D = kT/6\pi r\eta$). Olive oil/water partition coefficients were taken from Collander (1954). The partition coefficients for a few solutes were not available in the literature, and these we measured directly. Five μCi of each solute was shaken vigorously for 24 hr with a mixture of olive oil and water, and after separation of the layers, the activity in each phase was estimated by scintillation counting. The

Table 1. Unstirred layer thickness (μ)

Method	Mucosal (δ_1)	Serosal (δ_2)	Total ($\delta_1+\delta_2$)
Na/K bi-ionic diffusion potential	$70\pm10(5)$	$90\pm6(6)$	160
Na/K bi-ionic diffusion potential (ouabain present)	$80\pm0(5)$	$220\pm20(5)$	300
$P_{\text{Benzyl alcohol}}$	–	–	200

partition coefficients obtained were: 1,7-heptanediol, $0.03\pm0.00(5)$; 1,2-propanediol, $0.002\pm0.000(3)$; nicotinamide, $0.005\pm0.001(5)$; glycine, $0.0005\pm0.0001(5)$; benzyl alcohol, $1.67\pm0.00(3)$; isobutyramide, 0.014 and 0.013 (2 expts.). Values obtained by both Collander (1954) and ourselves closely agree.

The thickness of the unstirred layers at the mucosal and serosal surfaces of the epithelium was estimated from the time course of the build-up of Na/K bi-ionic diffusion potentials (*cf.* Dainty & House, 1966). In the calculation of the effective thickness of these layers it was assumed *i*) the mucosal and serosal surfaces of the tissue behaved as Na-selective and K-selective membranes, respectively, and *ii*) the solute diffusion coefficients in the unstirred layers are the free solution values (*cf.* Smulders & Wright, 1971). In addition, we estimated the total thickness of the unstirred layers from the permeability of benzyl alcohol assuming that the membrane offered no significant resistance to the flux of this molecule from the mucosal to serosal solutions. Under these conditions the relation

$$\frac{1}{P}=\frac{1}{P_m}+\frac{\delta}{D}$$

(where P is the observed permeability coefficient, P_m is the membrane permeability coefficient, D is the free solution diffusion coefficient, and δ is the thickness of the unstirred layer) reduces to

$$\frac{1}{P}=\frac{\delta}{D}.$$

In the case of benzyl alcohol, this assumption appears to be justified because 1) the olive oil partition coefficient is high (1.67, i.e., about 3 times greater than the second most lipophilic compound used), 2) the flux across the tissue showed a sharp exponential decay with time (*cf.* Smulders & Wright, 1971), and 3) the apparent activation energy for permeation was about 5 kcal/mole above 20° but about 26 kcal/mole below 15 °C (Bindslev & Wright, *in preparation*).

The thickness of the unstirred layers in the toad urinary bladder measured under identical conditions as the permeability coefficients is listed in Table 1. In the case of the electrical measurements, the total thickness of the unstirred layers ranged from 160 to 300 μ, with an average value of 200 μ. As this average value compared well with that calculated from the fluxes of benzyl alcohol across the bladder, we used this unstirred layer thickness to determine the membrane permeability coefficients for the solutes listed in Table 2. With permeability coefficients up to 500×10^{-7} cm/sec, the correction for unstirred layers amounted to less than 15% but for highly permeant solutes such as butanol and water diffusion across the unstirred layers was close to rate limiting. In fact, in the presence of ADH, the unstirred layers did become rate limiting for the flux of butanol and water across the toad bladder.

All calculations were performed on a programmed Hewlett Packard calculator (Model 9100a) or an APL 360 on-line computer. All errors are expressed as standard errors with the number of estimates in parentheses.

Results and Discussion

Permeability Coefficients

In each lobe of the urinary bladder, it was possible to measure two permeability coefficients, either the permeability of two different solutes or the permeability of one solute in the absence and presence of agents used to modify the permeability of the tissue. The permeability of two compounds was obtained by measuring the flux of one compound for 180 min and then washing the mucosal and serosal compartments free of the isotope before adding a second nonelectrolyte to the mucosal fluid. In this type of experiment, the electrical conductance of the bladder was used as an index of the viability of the preparation. Using this procedure, we have compared directly the permeability of each of the 23 solutes with several of the other 22 compounds. In this manner we have minimized variability among different preparations of the urinary bladder as a source of error in our results.

All permeability coefficients measured in unmodified urinary bladder are listed in Table 2 in order of increasing permeability. Also included in Table 2 is the effect of ADH on nonelectrolyte permeability. This is expressed as the ratio of the membrane permeability coefficients obtained in the presence and absence of ADH. Apart from benzoic acid, there was a significant increase in the permeability with ADH for all the compounds having permeability coefficients greater than 10×10^{-7} cm/sec. Only the amino acid, glycine, which has a net charge at physiological pH, showed a significant decrease ($p < 0.01$) in permeability in hormone-treated membrane [1].

Permeation of Lipophilic Solutes

For the purpose of discussion, molecules with olive oil partition coefficients significantly greater than water are considered as lipophilic molecules, and those with partition coefficients significantly less than water are consid-

1 With the mucosal solution at pH 6.0 (isoelectric point for glycine), P's for glycine, nicotinic acid and acetamide are not significantly different from controls (mucosa at pH 7.4). Treatment with ADH, however, significantly increases glycine P's to $210 \pm 20\%$ (12) of control ($p < 0.001$) with the mucosal solution at the pI of the amino acid. P_{ADH}'s for acetamide and nicotinic acid show no variation from the normal hormone response.

Table 2. Toad urinary bladder P's

Compound	P (cm/sec × 10^7)	P_m (cm/sec × 10^7)	$P_{m\ ADH}/P_m$
1. Sucrose	1.4±0.1(33)	1.4	1.0±0.1(18)
2. Mannitol	1.9±0.4(9)	1.9	0.9±0.5(8)
3. Galactose	2.0±0.4(3)	2.0	1.0±0.1(2)
4. Arabinose	2.2±0.3(3)	2.2	1.1±0.1(2)
5. Erythritol	3.0±0.2(26)	3.0	1.3±0.2(6)
6. α-Aminoisobutyric Acid	3.5±0.3(5)	3.5	1.0±0.0(4)
7. Nicotinic Acid	4.4±0.3(19)	4.4	0.9±0.1(15)
8. Glycine	9.2±0.4(20)	9.3	0.9±0.0(20)
9. Nicotinamide	12 ±1(8)	12	1.9±0.2(6)
10. Urea	14 ±1(16)	14	3.5±0.6(2)
11. Acetamide	16 ±1(44)	16	4.5±1.0(14)
12. Isobutyramide	16 ±1(20)	16	2.2±0.2(8)
13. 1,4-Butanediol	16 ±0(5)	16	1.7±0.0(2)
14. 1,2-Propanediol	16 ±1(20)	18	1.6±0.2(6)
15. Benzoic Acid	24 ±1(16)	24	1.1±0.1(8)
16. *n*-Butyramide	53 ±2(24)	54	1.6±0.2(12)
17. 1,6-Hexanediol	71 ±2(12)	72	1.5±0.1(8)
18. Antipyrene	96 ±10(10)	100	1.3±0.0(2)
19. 1,7-Heptanediol	250 ±10(40)	280	1.3±0.0(8)
20. Caffeine	260 ±10(6)	290	1.3±0.0(2)
21. Water	1000 ±60(56)	1300	>10(8)
22. *n*-Butanol	3000 ±400(4)	9100	>1.8(4)

P's were measured as described in the text and corrected for the presence of unstirred layers (i.e., P_m). Permeability coefficients in the presence of 130 mU ADH/ml are expressed relative to rates of permeation in the absence of hormone.

ered as hydrophilic molecules. P's across toad urinary bladder for the lipophilic solutes are directly proportional to K_{oil}, with a linear regression coefficient of 0.86 (*cf.* Fig. 1, Wright & Pietras, 1974).

The P's for nine lipophilic nonelectrolytes in the presence of ADH are plotted against those in the absence of ADH in Fig. 1. This shows that there is a linear relation between the permeabilities in the absence and presence of hormone. The slope of the line was 1.3, and the linear regression coefficient was 0.99. This evidence leads to the conclusion that the hormone produces a generalized, but unselective, increase in the permeability of all the lipophilic compounds. The permeability of virtually all the compounds increases by roughly the same factor, and there is no apparent change in the selectivity. The same intramolecular forces, therefore, which govern selectivity for lipophilic molecules in untreated toad bladder (Wright & Pietras,

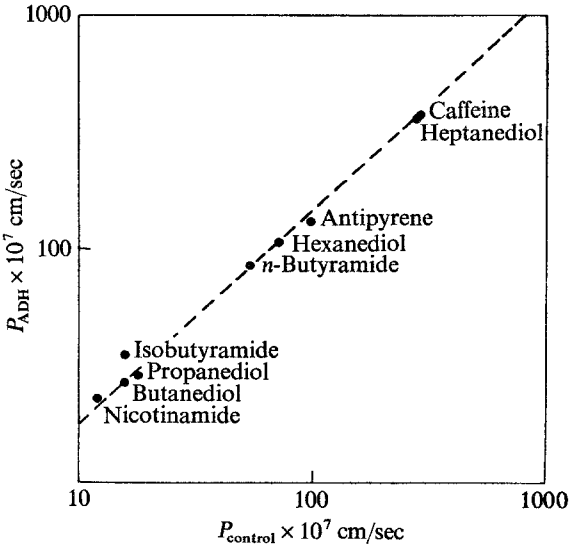

Fig. 1. Effect of ADH on the permeation of lipophilic solutes across toad urinary bladder. Membrane permeability coefficients (cm/sec) in the presence of 130 mU ADH/ml serosal medium are compared directly with coefficients in the absence of hormone

1974) are present in hormone-treated tissues. Analysis of the results using the approach introduced by Lieb and Stein (1969) yields the same conclusions.

Permeation of Branched-Chain Solutes

Many biological membranes discriminate between branched and straight-chain isomers (Diamond & Wright, 1969; Wright & Pietras, 1974). This has been attributed to the anisotropy of membrane lipids. Physical evidence indicates that the hydrocarbon tails of fatty acid residues in membranes have a more ordered configuration than that of bulk lipid solvent. A branched molecule is considered to produce a grater disruption of local lipid structure and to encounter more steric hindrance than a straight-chain molecule. In the toad bladder in the absence of ADH, paired experiments on tissue extracted from the same animal show that permeability of isobutyramide is $16 \pm 1(20) \times 10^{-7}$ cm/sec, whereas the permeability of *n*-butyramide is $54 \pm 3(20) \times 10^{-7}$ cm/sec. This difference in permeability is strikingly similar to that for the permeation of the amides across red cell membranes (Sha'afi, Gary-Bobo & Solomon, 1971) and the gallbladder epithelium (Wright & Pietras, 1974). It may thus be concluded that the membrane lipids of toad urinary bladder exist in a highly ordered configuration.

The permeability of both isomers increased when ADH was added. The increase with isobutyramide was much more dramatic than with the straight-chain isomer. The permeabilities of isobutyramide and *n*-butyramide increased to $35 \pm 3(8) \times 10^{-7}$ and to $68 \pm 7(8) \times 10^{-7}$ cm/sec, respectively. The ratio of the isobutyramide to *n*-butyramide permeabilities increased from $0.30 \pm 0(20)$ to $0.51 \pm 0(8)$ when the membrane was treated with ADH. (The ratio for K_{oil} is 0.82.) The increase was highly significant at $p < 0.001$. These results strongly suggest that the branched molecule encounters less steric hindrance in permeating across ADH-treated membranes than in control membranes. This leads to the conclusion that ADH increases the permeability of the bladder epithelium by increasing the fluidity of the cell membranes.

In both biological and artificial membranes, increases in membrane fluidity have been associated with significant increments in nonelectrolyte permeation (Wilson, Rose & Fox, 1970; Kroes & Ostwald, 1971). Furthermore, recent studies of liposomes show that the partition of solutes into the membrane decreases when the hydrocarbon tails of membrane lipids pass from a fluid into a crystalline state (McFarland, 1972; Diamond & Katz, 1974; Dix, Diamond & Kivelson, 1974). This suggests that ADH effects in toad bladder epithelium result from an increase in the partition of solutes into plasma membranes, but associated increases in solute mobility cannot be excluded.

Permeation of Hydrophilic Solutes

The effect of ADH on the permeability of the toad bladder to lipophilic solutes is much smaller than that obtained with the small polar solutes. For example, the *P*'s of urea and water increased from $14 \pm 1(16)$ and $1300 \pm 80(56)$ cm/sec $\times 10^{-7}$ to $49 \pm 8(2)$ and greater than 10,000(8) cm/sec $\times 10^{-7}$. On the other hand, the larger polar solutes (*see* sucrose, mannitol, galactose, arabinose and erythritol in Table 1) show no significant increments in permeability. The relationship between *P*'s (in the presence and absence of hormone) and molecular volume for the hydrophilic solutes is depicted in Fig. 2. As found previously in other epithelia (*cf.* Wright & Pietras, 1974), the results show that there are two components in the relationship between *P* and *MV* both in the presence and absence of hormone – one component for *MV* significantly less than 80 cc/mole and another for *MV* significantly greater than 80 cc/mole. The plots for *P*'s of the large polar solutes are essentially the same in the presence and absence of ADH. The slope of the relationship between log *P* and *MV* is -0.0026 with hormone and -0.0021 without hormone. For the smaller polar solutes (acetamide, urea and

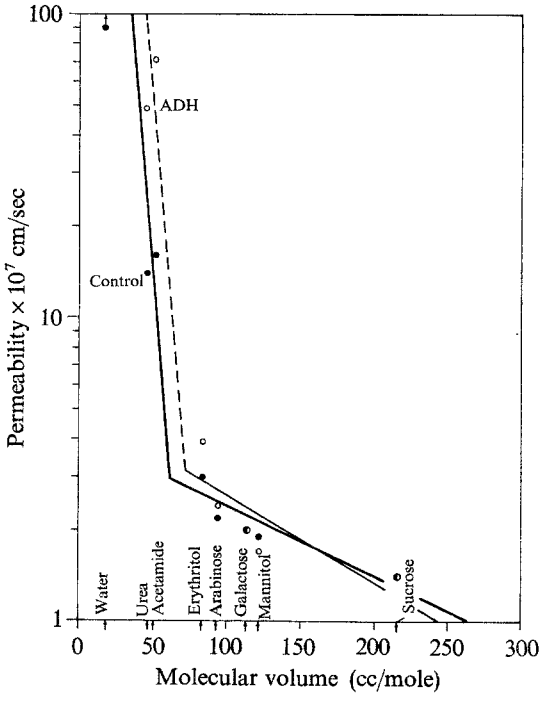

Fig. 2. Effects of ADH on the permeability of toad urinary bladder to hydrophilic solutes. Membrane permeability coefficients (cm/sec) are plotted as a function of molecular volume in the presence (○) and absence (●) of 130 mU ADH/ml serosal medium. The dotted line is the ADH curve for the small polar solutes (acetamide, urea, water) predicted from juxtaposition of the normal curve as described in the text

water), the slope of this relationship (in the absence of ADH) is much steeper. Since the unstirred layers become rate limiting for water transport in the presence of hormone, it is not possible to accurately determine the slope of the relationship between ADH P's and MV for the latter polar solutes. Extrapolation of the ADH curve, however, from juxtaposition of the normal curve predicts that water permeability in the presence of ADH will approach $10{,}000 \times 10^{-7}$ cm/sec, as was also indicated from our unstirred layer correction. Thus, as suggested by other investigators (*see* Hays, 1972), the increment in diffusional water permeability is much larger than was previously suggested. Furthermore, it is quite possible that this increase may be compatible with the large hormone-dependent increment in osmotic water permeability in the toad bladder.

Collectively, the data lead to the conclusion that ADH acts to increase permeability across the native lipid component of the cell membrane, considered to be the preferred pathway for movement of lipophilic and

small polar solutes (Bindslev & Wright, 1974; Wright & Pietras, 1974). The notable lack of a hormone effect on the permeation of large hydrophilic solutes suggests that ADH does not alter extracellular pathways.

Comparison of Membrane Effects of ADH and cAMP

Handler & Orloff (1973) have reviewed evidence that cAMP mimics the effect of ADH on water and sodium transport. They suggest that ADH-induced nucleotide may function as the intracellular mediator of hormone action. In view of this evidence, we compared the effects of cAMP and ADH on SCC, conductance (G) and P's for a series of eight lipophilic and hydrophilic nonelectrolytes.

We find that short-circuit current (SCC) in the presence of cAMP peaks at $210 \pm 20\%(5)$ of controls within 10 min. This measurement was not significantly different from the maximal ADH-induced SCC, $250 \pm 20\%(25)$ of controls, recorded 10–15 min following hormone addition to the serosal medium. ADH (130 mU/ml) and cAMP (10 mM), therefore, elicit identical increments in SCC. These data are consistent with previous reports.

On the other hand, conductance changes measured in the 90-min period following addition of ADH or cAMP were significantly different [$p < 0.001$ (160)] from each other. G in cAMP-treated bladders rose to $120 \pm 0\%(35)$ of controls during the first hour but declined to $100 \pm 10\%(10)$ of controls by 90 min. In contrast, the G of hormone-stimulated bladders increased to $140 \pm 0\%(95)$ of controls by hour one and continued to climb to $150 \pm 0\%(20)$ of controls by 90 min. Cuthbert and Painter (1969) have also reported a divergence in the effects of cAMP and ADH on G in isolated frog skin.

The effects of cAMP on nonelectrolyte transport are presented as the permeability ratio, P_{cAMP}/P_{ADH}, in Fig. 3. All P's except those for heptanediol and arabinose are significantly different from control P's at $p < 0.05$. The results indicate that there are both qualitative and quantitative differences in the patterns of nonelectrolyte selectivity in the presence of hormone as compared to cAMP. For all compounds except arabinose, ADH P's are significantly different from cAMP P's. Although selected concentrations of ADH and cAMP produce effects on SCC which are not significantly different, the respective changes in nonelectrolyte transport are markedly divergent.

The most dramatic effect of cAMP on membrane transport is the enhanced permeability of large polar solutes. Such compounds are considered to permeate predominantly via "shunts"; i.e., a few large pores in the tight

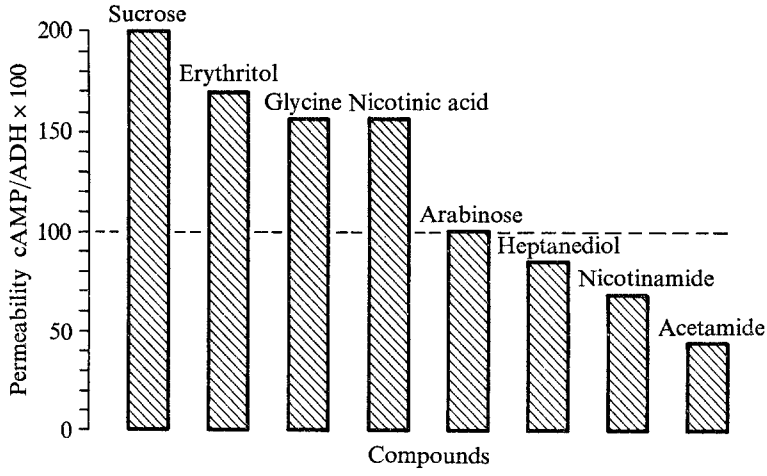

Fig. 3. Comparison of membrane effects of ADH and cAMP. Membrane permeability coefficients (cm/sec × 10^7) obtained in the presence of 10 mM cAMP are $2.8 \pm 0.3(16)$, sucrose; $6.6 \pm 0.9(4)$, erythritol; $13 \pm 1(6)$, glycine; $6.2 \pm 0.1(6)$, nicotinic acid; 2.4(2), arabinose; $308 \pm 30(6)$, heptanediol; $16 \pm 0(6)$, nicotinamide; and $32 \pm 6(6)$, acetamide. These individual *P*'s are expressed relative to coefficients measured under the same conditions but in the presence of 130 mU ADH/ml serosal medium (*see* Table 2)

junctions (Wright & Pietras, 1974). Effects of cAMP on the contractile activity of submucosal smooth muscle in toad bladder (Eggena, Schwartz & Walter, 1968) may bear on the rate of transport through these extracellular pathways. Parisi, Ripoche and Bourguet (1969) have shown that sheets of frog urinary bladder epithelial cells isolated from smooth muscle are fully responsive to neurohypophyseal hormones, whereas the response to cAMP was markedly reduced, and, in most experiments, theophylline failed to potentiate hormone action.

cAMP effects on the transport of small hydrophilic molecules (i.e., acetamide) and lipophilic molecules (i.e., heptanediol) which probably permeate through the cell membrane (Wright & Pietras, 1974) are much less marked. Other have also noted that the effects of exogenous cAMP on osmotic water flow in toad bladder are relatively minor (Wong, Bedwani & Cuthbert, 1972). The antidiuretic effect of neurohypophyseal hormones in kidney tissue occurs in mammalian and avian species. Stimulation of hormone-induced adenylate cyclase, however, has only been detected in mammalian kidney. Adenylate cyclase from kidney of pigeon shows no response to vasopressin, oxytocin or 8-arginine vasotocin (Dousa, Walter, Schwartz, Sands & Hechter, 1972). Furthermore, in toad bladder, the response of the adenylate cyclase is maximal at ADH concentrations far lower than those

required for the greatest effect on water flow (Wong *et al.*, 1972). On the other hand, the activity of hormone-induced adenylate cyclase and consequent cAMP generation do show a good quantitative and temporal relationship to net sodium transport in toad urinary bladder (Sapirstein & Scott, 1973). It may also be pertinent here to note that angiotensin II, an octapeptide similar to ADH, enhances osmotic water transport in isolated toad bladder (Coviello, 1973), but angiotensin-amide does not elevate cAMP concentration in this tissue (Handler, Butcher, Sutherland & Orloff, 1965). Independent actions on sodium and nonelectrolyte transport, respectively, across bladders treated with ADH have been suggested previously (*cf.* Petersen & Edelman, 1964). The divergence in the nonelectrolyte transport effects of ADH and cAMP at concentrations which elicit equivalent increments in SCC lends support to these earlier assumptions.

Influence of Surface Charge on Membrane Effects of ADH

It is known that ADH selectively alters the permeability (Maffly, Hays, Lamdin & Leaf, 1960; Leaf & Hays, 1962) and conductance (Civan & Frazier, 1968) of mucosal membrane but is ineffective when applied directly to the mucosal surface. In view of reports that ADH can similarly enhance permeability (Fettiplace, Hayden & Knowles, 1971; Graziani & Livine, 1971; Kafka & Pak, 1972) and conductance (Bach & Miller, 1974) of some artificial lipid membranes, it is important to determine what may prevent the action of ADH directly on the mucosal surface in biological membranes. The molecular structure of ADH contains many ionizable groups and dipole moments which may be important determinants of hormone interaction with the membrane surface. Most epithelial cell membranes are electronegative and a divergence of basal/apical surface charge has been found in several cell populations. Thus charge interactions between the hormone and the surface of the epithelial membrane could play an important role in governing the effect of ADH on the toad bladder.

We decided to test this by modifying the magnitude of the surface potential on the mucosal side of bladder with the trivalent cation, La^{3+}. Since the surface charge density of toad bladder is sufficiently high (Lipman, Dodelson & Hays, 1966), La^{3+} should reduce the surface potential by a screening mechanism (McLaughlin, Szabo & Eisenman, 1971). In these experiments, 0.5 mM $LaCl_3$ was added to the mucosal solution, and SCC, G and nonelectrolyte P's (water, urea, heptanediol and antipyrene) were monitored for 90 min before the addition of hormone. During this time period, P's and G are not significantly different from control measurements.

Table 3. Effects of serosal ADH

	ADH/Control	
	0 La^{3+}	0.5 mM La^{3+}
G	1.4±0.0(18)	1.4±0.0(23)
P_{HOH}	>7.0 (6)	4.7±0.4(14)
P_{Urea}	3.5±0.6(3)	2.9±0.3(3)
$P_{Heptanediol}$	1.3 (2)	1.3 (2)
$P_{Antipyrene}$	1.3 (2)	1.3 (2)

Permeability (P) and conductance (G) measurements with 130 mU ADH/ml serosal solution in the presence and absence of 0.5 mM $LaCl_3$ (mucosal) are expressed relative to equivalent parameters measured under the same conditions but in the absence of hormone.

Table 4. Effects of mucosal ADH

	ADH/Control	
	0 La^{3+}	0.5 mM La^{3+}
SCC	1.0±0.0(3)	1.5±0.0(3)
G	1.1±0.1(50)	1.3±0.0(23)
P_{HOH}	1.1±0.1(3)	1.7±0.2(10)
P_{Urea}	1.0 (2)	1.3±0.1(3)
$P_{Heptanediol}$	1.0±0.0(4)	1.3 (2)
$P_{Antipyrene}$	1.0 (2)	1.3 (2)

Various permeability (P) and electrical measurements (SCC, G) with 130 mU ADH/ml mucosal medium in the presence and absence of 0.5 mM $LaCl_3$ are expressed relative to the same parameters measured under the same conditions but in the absence of hormone.

SCC, however, transiently increases to 160±10% of controls within 30 min but returns to baseline levels by 60 min.

The effects of serosal ADH in the presence and absence of mucosal $LaCl_3$ are presented in Table 3. The membrane response to hormone in the presence of La^{3+} is not significantly different from the response in the absence of La^{3+}, but the increment in P_{water} under these conditions appears to be depressed with La^{3+}.

The effects of mucosal ADH in the presence and absence of mucosal $LaCl_3$ are presented in Table 4. As reported previously (e.g. Leaf & Hays, 1962), addition of hormone to the mucosal medium has no significant effect. On the other hand, mucosal ADH in the presence of mucosal La^{3+} elicits increments in P's, G and SCC which are significantly different from controls (absence of mucosal ADH) at $p < 0.05$. These latter effects on P's

for water, urea and on G, however, are significantly different from the effects of serosal ADH with La^{3+} present in the mucosal solution. Only the P's for heptanediol and antipyrene, the lipophilic probes, are not significantly different. Although the mucosal ADH P's for water and urea are some 50–60% less than serosal ADH P's, the ratio of P_{water} to P_{urea} remains virtually unchanged. The SCC induced by mucosal ADH, however, is both quantitatively ($p < 0.001$) and qualitatively different from the serosal ADH control. We find that the normal hormone-induced SCC rises to a peak approximately 10–15 min following addition of ADH serosally, and then falls toward baseline levels. Following mucosal ADH, however, SCC rises to a peak level within 7 min and remains elevated during the entire 60-min observation period.

These data show that ADH can elicit changes in nonelectrolyte transport by direct interaction with mucosal membrane[2]. In some respects, these permeability effects mimic the serosal action of hormone, but the change in SCC appears to be divergent from the normal response.

Conclusions

The results of these experiments show clearly that (1) ADH does not enhance the permeability of large hydrophilic solutes considered to permeate via extracellular pathways, but (2) ADH does enhance the permeability of lipophilic and small polar solutes. We have previously suggested that water and other small solutes, as well as the lipophilic compounds, may penetrate the native lipid component of the cell membrane (Wright & Pietras, 1974). The anomalous permeation of these small solutes may be, at least in part, due to the highly ordered structure of the membrane lipids and extremely low molecular volume of these compounds (*see also* Bindslev & Wright, 1974). It is not surprising, therefore, that the water permeability and ADH-responsiveness of certain lipid bilayer membranes are strikingly similar to that for biological membranes (Fettiplace *et al.*, 1971; Graziani & Livne, 1971; Kafka & Pak, 1972).

The differential effect of ADH on the permeability of structural isomers further suggests that the hormone acts to enhance the motional freedom or

2 From measurements of the transepithelial permeability of ^{125}I-arginine vasopressin in toad bladder, we have found that the rate of hormone penetration is conceivably fast enough to allow movement from mucosal to serosal surfaces in the time span of the above observations. This explanation of the observed effects of mucosal ADH, however, is excluded by consideration of the fact that (1) mucosal hormone in the absence of membrane-bound La^{3+} has no effect on membrane transport and (2) the membrane response to mucosal ADH in the presence of La^{3+} is substantially different in several respects from the response to serosal hormone.

fluidity of membrane lipids[3]. Such changes in membrane fluidity could produce quantitatively different effects on lipophilic and hydrophilic solutes. 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 situated nearer the polar head groups of the lipids (Diamond & Katz, 1974). If, for example, ADH produced a larger increment in fluidity at the core than at the periphery of the bladder membranes, there should be a greater increase in the permeability of hydrophilic solutes than lipophilic solutes.

Blockade of the hydro-osmotic effect of ADH in bladder by anesthetics (Gray & Ullmann, 1969) and other agents (Taylor, Mamelak, Reaven & Maffly, 1973) may well be attributable to counteractive influences on the plasma membrane (*see* Roth, 1973). Our experiments also suggest that electrostatic interactions of ADH with charged groups at the membrane periphery can strongly influence hormone action. Reduction of the mucosal surface potential with La^{3+} enabled a direct interaction between ADH and apical membrane. Such data point out the importance of the physical characteristics of the plasma membrane in determining hormone action.

Although selected concentrations of ADH and cAMP elicit equivalent increments in SCC, the respective effects on nonelectrolyte permeability and G are divergent. These findings may be related to other reports of conditions in which the nonelectrolyte permeability response to ADH can be abolished or reduced while the response of the adenylate cyclase system and SCC is unimpaired (Petersen & Edelman, 1964; Argy, Handler & Orloff, 1967; Cuthbert & Painter, 1968; Lipson & Sharpe, 1971; Wong *et al.*, 1972). Although ADH affects cellular biochemistry via adenylate cyclase, it may also trigger other concurrent or sequential events which could mediate or facilitate the hormone effect. The clear divergence of nonelectrolyte movement elicited by ADH and cAMP, at concentrations which stimulate SCC equally, suggests further that the mechanism of hormone action on nonelectrolyte permeation and sodium transport may be different.

We wish to thank Drs. N. Bindslev and J. M. Diamond for valuable discussions and comments on the manuscript. This work was supported by a USPHS Research Grant (NS-09666). R.J.P. was supported by a Mental Health Training Grant (5 T01-MH 06415-16).

3 Positive, ADH-induced increments in lectin-mediated hemagglutination of epithelial cells isolated from toad urinary bladders, in the absence of quantitative changes in lectin binding sites, lends further support to this conclusion (Pietras, Naujokaitis and Szego, *in preparation*).

References

Argy, W. P., Handler, J. S., Orloff, J. 1967. Calcium and magnesium effects on toad bladder response to cyclic AMP, theophylline and ADH analogues. *Amer. J. Physiol.* **213**:803

Bach, D., Miller, I. R. 1974. Interaction of vasopressin with phosphatidylserine bilayers. *Biochim. Biophys. Acta* **339**:367

Bindslev, N., Wright, E. M. 1974. Anomalous permeation of small lipophilic solutes across frog urinary bladder. *J. Physiol. (In press)*

Civan, M. M., Frazier, H. S. 1968. The site of the stimulatory action of vasopressin on sodium transport in toad bladder. *J. Gen. Physiol.* **51**:589

Collander, R. 1954. The permeability of *Nitella* cells to nonelectrolytes. *Physiol. Plant.* **4**:420

Coviello, A. 1973. Hydroosmotic effect of angiotensin II in the toad bladder. *Acta Physiol. Latin Amer.* **23**:350

Cuthbert, A. W., Painter, E. 1968. Independent action of antidiuretic hormone, theophylline and cyclic AMP on cell membrane permeability in frog skin. *J. Physiol.* **199**:593

Dainty, J., House, C. R. 1966. Unstirred layers in frog skin. *J. Physiol.* **182**:66

Diamond, J. M., Katz, Y. 1974. Interpretation of nonelectrolyte partition coefficients between dimyristoyl lecithin and water. *J. Membrane Biol.* **17**:121

Diamond, J. M., Wright, E. M. 1969. Biological membranes: The physical basis of ion and nonelectrolyte selectivity. *Annu. Rev. Physiol.* **31**:581

Dix, J. A., Diamond, J. M., Kivelson, D. 1974. Translational diffusion coefficient and partition coefficient of a spin-labelled solute in lecithin bilayer membranes. *Proc. Nat. Acad. Sci. U.S.A.* **71**:474

Dousa, T. P., Walter, R., Schwartz, I. L., Sands, H., Hechter, O. 1972. Role of cyclic AMP in the action of neurohypophyseal hormones on kidney. *In:* Advances in Cyclic Nucleotide Research. P. Greengard, R. Paoletti and G. A. Robison, editors. Vol. I, p. 121. Raven Press, New York

Eggena, P., Schwartz, I. L., Walter, R. 1968. Effects of neurohypophyseal hormones, theophylline and nucleotides on the smooth muscle of toad bladder. *Life Sci.* **7**:979

Fettiplace, R., Haydon, D. A., Knowles, C. D. 1971. The action of lysine vasopressin on artificial lipid monolayers. *J. Physiol.* **221**:18P

Graziani, Y., Livne, A. 1971. Vasopressin and water permeability of artificial lipid membranes. *Biochem. Biophys. Res. Commun.* **45**:321

Grey, D., Ullmann, E. 1969. Reduction by anesthetics of the effect of ADH on permeability of toad bladder. *J. Physiol.* **200**:66P

Handler, J. S., Butcher, R. W., Sutherland, E. W., Orloff, J. 1965. The effect of vasopressin and of theophylline on the concentration of adenosine 3′,5′-phosphate in urinary bladder of toad. *J. Biol. Chem.* **240**:4524

Handler, J. S., Orloff, J. 1973. The mechanism of action of antidiuretic hormone. *In:* Handbook of Physiology—Renal Physiology. S. R. Geiger, J. Orloff and R. W. Berliner, editors. Section 8, p. 791. The Williams & Wilkins Co., Baltimore, Md.

Hays, R. M. 1972. The movement of water across vasopressin-sensitive epithelia. *In:* Current Topics in Membranes and Transport. F. Bronner and A. Kleinzeller, editors. Vol. 3, p. 339. Academic Press, New York

Kafka, M. S., Pak, C. Y. C. 1972. Effect of polypeptide hormones on lipid monolayers. *J. Colloid Interface Sci.* **41**:148

Kroes, J., Ostwald, R. 1971. Erythrocyte membranes—Effect of increased cholesterol content on permeability. *Biochim. Biophys. Acta* **249**:647

Leaf, A., Hays, R. M. 1962. Permeability of isolated toad bladder to solutes and its modification by vasopressin. *J. Gen. Physiol.* **45**:921

Lieb, W. R., Stein, W. D. 1969. Biological membranes behave as non-porous polymeric sheets with respect to the diffusion of nonelectrolytes. *Nature* **224**:240

Lipman, K. M., Dodelson, R., Hays, R. M. 1966. The surface charge of isolated toad urinary bladder epithelial cells. *J. Gen. Physiol.* **49**:501

Lipson, L. C., Sharpe, G. W. G. 1971. Effect of prostaglandin E_1 on sodium transport and osmotic water flow in toad bladder. *Amer. J. Physiol.* **220**:1046

Maffly, R. H., Hays, R. M., Lamdin, E., Leaf, A. 1960. The effect of neurohypophyseal hormones on the permeability of the toad bladder to urea. *J. Clin. Invest.* **39**:630

McFarland, B. G. 1972. Molecular basis of fluidity in membranes. *Chem. Phys. Lipids* **8**:303

McLaughlin, S. G. A., Szabo, G., Eisenman, G. 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J. Gen. Physiol.* **58**:667

Parisi, M., Ripoche, P., Bourguet, J. 1969. The isolated epithelium of the frog urinary bladder; responses to oxytocin, 3'5'-adenosine monophosphate and theophylline. *Pflüg. Arch.* **309**:59

Petersen, J. M., Edelman, I. S. 1964. Calcium inhibition of the action of vasopressin on the urinary bladder of the toad. *J. Clin. Invest.* **43**:583

Pietras, R. J., Wright, E. M. 1974*a*. Nonelectrolyte probes of membrane structure in ADH-treated toad urinary bladder. *Nature* **247**:222

Pietras, R. J., Wright, E. M. 1974*b*. Effects of mucosal ADH on toad urinary bladder. *Fed. Proc.* **33**:216

Roth, S. 1973. A molecular model for cell interactions. *Quart. Rev. Biol.* **48**:541

Sapirstein, V. S., Scott, W. N. 1973. Cyclic AMP and sodium transport. *J. Clin. Invest.* **52**:2379

Sha'afi, R. I., Gary-Bobo, C. M., Solomon, A. K. 1971. Permeability of red cell membrane to small hydrophilic and lipophilic solutes. *J. Gen. Physiol.* **58**:238

Smulders, A. P., Wright, E. M. 1971. The magnitude of nonelectrolyte selectivity in the gallbladder epithelium. *J. Membrane Biol.* **5**:297

Taylor, A., Mamelak, M., Reaven, E., Maffly, R. 1973. Vasopressin: Possible role of microtubules and microfilaments in its action. *Science* **181**:347

Wartiovarra, V., Collander, R. 1960. Permeabilitätstheorien. *Protoplasmatologia 2c 8d*

Wilson, G., Rose, S. P., Fox, C. F. 1970. The effect of membrane lipid unsaturation on glycoside transport. *Biochem. Biophys. Res. Commun.* **38**:617

Wong, P. Y. D., Bedwani, J. R., Cuthbert, A. W. 1972. Homone action and the level of cyclic AMP and prostaglandins in the toad bladder. *Nature, New Biol.* **238**:27

Wright, E. M., Barry, P. H., Diamond, J. M. 1971. The mechanism of cation permeation in rabbit gallbladder. *J. Membrane Biol.* **4**:331

Wright, E. M., Pietras, R. J. 1974. Routes of nonelectrolyte permeation across epithelial membranes. *J. Membrane Biol.* **17**:293