Permeability of the Isolated Toad Bladder to Solutes and Its Modification by Vasopressin

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ABSTRACT Measurements have been made of the permeability of the isolated urinary bladder of the toad to a number of small solute molecules, in the presence and absence of vasopressin. Vasopressin has a strikingly specific effect on increasing permeability of the bladder to a group of small, uncharged amides and alcohols while penetration by other small molecules and ions is unaffected. The movement of urea is passive, as indicated by equal flux rates in the two directions. The reflection coefficients for chloride and thiourea indicate a high degree of impermeability of the bladder to these solutes even in the presence of large net movements of water. The low concentration of thiourea in the tissue water when this compound is added to the mucosal bathing medium indicates that the major permeability barrier to thiourea is at the mucosal surface of the bladder. The findings can be accounted for by a double permeability barrier consisting of a fine selective diffusion barrier and a porous barrier in series. The former would constitute the permeability barrier to most small solutes while the latter would be the rate-limiting barrier for water and the amides. It would be the porous barrier which is affected by vasopressin. Reasons are presented which require both barriers to be contained in or near the plasma membrane at the mucosal surface of the bladder.

INTRODUCTION

In the preceding paper (1) are presented studies on the movement of water through the isolated urinary bladder of the toad and the influence of mammalian antidiuretic hormone upon this process. The picture derived from such studies is that of a porous membrane in which neurohypophyseal hormone accelerates net water transport by increasing the size of pores at the mucosal surface. This interpretation is in accord with the results and views of Koefoed-Johnsen and Ussing (2).

This paper reports the effects of neurohypophyseal hormone on the permeability of the toad bladder to solutes in the presence and absence of net water movement. The findings indicate a low degree of permeability of the membrane to most small solutes in spite of the large net transfers of water which may occur.

METHODS

The technique and calculations used for determining isotopic permeability coefficients have been described (3, 4). Briefly, the tagged molecule or ion tested is added to the medium bathing one surface of the toad bladder mounted between two halves of a lucite chamber. From the rate of appearance of isotope in the medium bathing the opposite surface and its concentration in the medium on the side to which it was originally added, the permeability coefficients, K_{trans}, is readily calculated. The cross-sectional area of the chamber was 7.07 cm^2 and the volume of Ringer's solution bathing each surface was equal at 15 to 20 ml. Except as indicated, the test substance was used at the specific activity at which it was received from the supplier. When permeability of the bladder was examined in the presence of net water movement, the latter was produced and measured by the methods described in the preceding paper (1). Commercial vasopressin (Parke Davis & Company) was the neurohypophyseal hormone used. The methods for preparing and measuring the N^{15}-urea used in the simultaneous flux measurements with C^{14}-urea have been described (4). All transmembrane permeability coefficients, K_{trans}, are averages of at least two successive 30 minutes of measurement before and after addition of 1 to 2 units of vasopressin to the serosal bathing medium.

The sources of the radioactive chemicals used in this study were: Water labeled with tritium and the following C^{14}-labeled molecules: methanol, ethanol, ethylene glycol, urea, acetamide, propionamide, butyramide, methane, nicotinamide, methylacetamide, thiocyanate, methylsulfate, lactate, glycine, glycylglycine, guanidine, acetonitrile, thiourea (both C^{14} and S^{35}) acetanilide, glycerol, butanol, inulin, and sucrose were obtained from New England Nuclear Corporation, Boston, Formate, salicylate, ethanolamine, formaldehyde, and cyanamide (all C^{14}-labeled) were obtained from Volk Radio-Chemical Company, Chicago. C^{14}-choline and C^{14}-arginine were from Nuclear-Chicago Corporation, Chicago, C^{14}-dimethylformamide and C^{14}-propylene glycol were obtained from Orlando Research Inc., Orlando, Florida. $P^{32}O_4$, K^{42}, and Na^{24} were from Brookhaven National Laboratories and Cl^{36} and $S^{35}O_4$ from Oak Ridge National Laboratories. 1,5-sorbitan and 3-methylglucose (tritium-labeled) were kindly made available to us by Dr. Robert K. Crane, Washington University, St. Louis.

RESULTS

Penetrability Unaffected by Hormone

Table I lists the molecules and ions for which the permeability of the toad bladder has been examined and found to be unaffected by mammalian

neurohypophyseal hormones. The absolute value of the transmembrane permeability coefficient is probably influenced much more for a permeating molecule by the lipid solubility of the compound than by its size. The more polar compounds as a group possess lower permeability coefficients. Although

TABLE I

COMPOUNDS NOT PENETRATING TOAD BLADDER MORE RAPIDLY WITH VASOPRESSIN

Species	Atomic or molecular weight	No. of experiments	Permeability coefficients K_{trans} (10^{-7} cm/sec.) Vasopressin			
			Before		After	
			Mean	Range	Mean	Range
Inorganic ions						
Sodium (S to M)	23	14	2.8	0.7–11	2.9	1.2–8.6
Potassium	39	12	26	5.2–51	29	4.2–80
Chloride	36	8	13	2.8–30.3	10	2.2–23
Sulfate	96	10	4.2	0.9–6.1	4.0	1.1–6.2
Phosphate	79	3	1.9	0.9–3.7	2.5	0.7–4.1
Organic ions						
Thiocyanate	58	1	8.3		8.6	
Methylsulfate	111	2	4.8	1.4–9	3.6	1.4–6
Formate	45	2	27	12–42	20	11–29
Choline	121	6	9.1	4.9–17	9.5	4–16
Lactate	89	12	5.6	2.1–13	5.5	2.7–18
Glycine	75	2	2.2	1.2–3.2	2.6	1.1–4.0
Glycylglycine	149	2	15	7.6–22	12	4.2–19
Arginine	174	2	1.8	1.7–2.0	1.3	1.3–1.4
Guanidine	59	3	1.5	0.6–2.9	1.8	0.5–2.6
Salicylate	138	2	14	14–14	6.2	3.7–8.6
Ethanolamine	61	2	11	8.3–14	6.3	4.8–7.8
Organic molecules						
Formaldehyde	30	4	251	224–306	229	214–257
Acetonitrile	41	2	1204	1100–1300	903	825–982
Thiourea	76	13	13.9	4.7–27	14.0	5.9–35
Acetanilide	135	2	927	897–983	917	866–1026
Glycerol	92	4	4.1	1.6–8.8	4.3	4.0–7.6
Butanol	74	2	930	766–1233	965	670–1200
1,5-Sorbitan	182	3	1.8	0.9–3.0	1.4	0.5–2.9
3-Methylglucose	195	1	8.2		8.6	
Sucrose	342	2	8.9	3.3–14	5.1	5–5.1
Inulin	5500	10	0		0	

we had previously found C^{14}-carboxy inulin to penetrate at a low and variable rate (4), in a recent group of ten experiments no detectable radioactivity penetrated the bladder in a period of 2 hours although the activity on one side of the bladder was 2 to 4 $\times$ 10^5 counts per minute per ml of medium. The bladder therefore appears to be essentially impermeable to a molecule

the size of inulin and our earlier findings probably resulted from overstretching or traumatizing the tissue slightly while mounting it or from loss of the C^{14}-label from the inulin.

All permeability measurements were made in the absence of net water movement. When permeability was measured in the two directions across the bladder equal values were obtained with the exception of sodium which is actively transported from the mucosal to serosal side (3). The spread of values indicated by the range reflects largely the variation from bladder to bladder. The values for the four 30 minute periods on each tissue were quite consistent.

Penetrability Increased by Hormone

For the smaller group of compounds listed in Table II a significant increase in the permeability coefficient resulted from addition of hormone. The only ion whose rate of penetration is affected by hormone is sodium and only the active transport from mucosal to serosal surfaces appears to be stimulated (5). Permeability to water is also increased by hormone, as discussed (1).

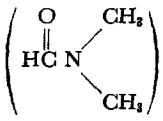
With the exception of the alcohols, all the other molecules listed in Table II possess in common an amide group or amide-like grouping (cyanamide). Because of the possibility of hydrolysis of cyanamide to urea and subsequent penetration by this compound the medium from both sides at the end of two experiments was tested for the presence of urea. This was accomplished by treating the medium with buffered urease, acidifying, and aerating to drive off any $C^{14}O_2$ which might have been formed. Even in the presence of considerable added non-radioactive urea such treatment failed to result in a detectable reduction in radioactivity of the media. Hence it was concluded that cyanamide penetrated the bladder without hydrolysis to urea.

Although possession of an amide group is common to many of the compounds in this group, its possession is not assurance of an augmented penetrability with hormone. The amino acids so far tested are examples and the failure of the hormone to affect their rates of penetration is probably related to their ionic character. However, even an uncharged amide such as acetanilide may fail to show a hormonal effect. In this case the high resting permeability coefficient probably is indicative of lipoid solubility and the absence of a detectable hormonal effect may be the consequence of the compound penetrating the bladder largely by non-aqueous routes. The same factors probably account for the increasing values of the resting permeability coefficients in the small series: acetamide, propionamide, and butyramide, and at the same time a decreasing response to hormone.

Even in the small group of alcohols tested this same trend seems apparent. The resting permeability coefficient of the small methanol molecule approaches that of water while ethanol penetrates at a lower rate. The larger

TABLE II

COMPOUNDS PENETRATING TOAD BLADDER MORE RAPIDLY WITH VASOPRESSIN

	Molecular weight	Permeability coefficients K_{trans} (10^{-7} cm/sec.) Vasopressin			
		Before	After	SE mean difference	*n*
Amides					
Urea (NH_2CNH_2)	60	26	274	±5	37
Acetamide (CH_3CNH_2)	59	44	196	±26	9
Propionamide ($CH_3CH_2CNH_2$)	73	97	215		2
Butyramide ($CH_3CH_2CH_2CNH_2$)	87	132	180		4
Cyanamide ($NH_2C{\equiv}N$)	42	127	282	±31	6
Urethane ($NH_2COCH_2CH_3$)	89	581	639	±18	6
Dimethylformamide 	73	174	259	±10	6
Nicotinamide ($C_5H_4NCNH_2$)	122	26	40	±2	6
Methylacetamide ($CH_3C\ NHCH_3$)	73	87	242	±16	6
Water and alcohols					
Water (HOH)	18	944	1580	±35	8
Methanol (CH_3OH)	32	825	913	±19	9
Ethanol (CH_3CH_2OH)	46	575	678	±20	8
Ethylene glycol (CH_2OHCH_2OH)	62	16	35	±6	6
Inorganic ions					
Sodium	23	36	52	±2.7	14

but less polar butanol (Table I) penetrates more rapidly but the small effect of hormone, still evident with the smaller alcohols, is no longer detectable with butanol. An increase in the polar nature of the alcohol, as with ethylene glycol, brings back the hormonal effect although probably molecular size reduces the resting permeability coefficient of this compound. Addition of another alcohol grouping to give glycerol results in further reduction in the permeability coefficient but also loss of the hormonal effect.

TABLE III
PERMEABILITY OF TOAD BLADDER
IN PRESENCE OF ACETAMIDE

Compound	Concentration acetamide	Molar ratio*	Vasopressin	
			Before‡ K_{trans}	After‡ (10^{-7} cm/sec.)
	M			
Dimethylformamide	0§		174	259
	0.1	300	222	370
	0.2	600	164	306
	0.4	1200	192	296
	0.6	1800	156	291
Nicotinamide	0§		25.2	39.1
	0.2	1800	12.0	21.7
	0.4	3600	20.2	34.2
	0.6	11,000	13.9	19.2
	0.6	11,000	24.8	39.5

* Molar ratio of acetamide to dimethylformamide or nicotinamide.
‡ Each value of K_{trans} is mean of two periods before and two periods after addition of vasopressin.
§ Mean K_{trans} of six experiments each.

Such considerations of the permeability coefficients of the compounds listed in Tables I and II in general support the thesis that neurohypophyseal hormones affect aqueous channels in the membrane. The marked difference in hormonal effects upon seemingly closely related molecules must await explanation until we possess much more detailed information regarding the composition and structure of living membranes.

Mode of Penetration

The permeability of the bladder from mucosal to serosal surface and from serosal to mucosal surface has been simultaneously determined with C^{14}- and N^{15}-labeled urea (4). Equal fluxes in the two directions were found with or without hormone indicating that urea moves passively through the membranes. The absence of active transport does not exclude the possibility of

specific interaction between urea and membrane as might occur if a carrier mediated the passage of urea. However no self-depression of the rate of penetration by radioactive urea could be detected when non-labeled urea was added to the medium in concentrations up to 0.1 molar. Higher concentrations of urea unfortunately could not be tested as they adversely affected the viability of the bladder.

Table III shows the negative results obtained when evidence for carrier-mediated penetration was sought by measurement of permeability of the bladder to C^{14}-labeled dimethylformamide and nicotinamide in the presence of increasing concentrations of non-radioactive acetamide. Even in the presence of high concentrations and large molar ratios of acetamide no detectable competition was noted.

Although we have been unable to demonstrate self-depression or competition for possible carrier sites, interaction with the membrane by hydrogen bonding is not excluded by these observations. Since it seems that specificity must require some unique interaction of these compounds with the tissue, we find it difficult to conclude that these compounds can pass through the bladder simply by free diffusion.

Solvent Drag on Urea

Andersen and Ussing have indicated (6) that when water moves by bulk flow, molecules caught up in the moving stream will be accelerated in the direction of flow and retarded in the direction opposite to flow. This effect has been termed "solvent drag." Fig. 1 shows that when the two unidirectional fluxes, J_1 and J_2, are measured simultaneously for urea in the presence of a net transfer of water imposed by an osmotic gradient across the bladder, the fluxes are rendered unequal. Theory requires (6) in a homoporous membrane that when the logarithm of the flux ratio of solute is plotted against the net water movement, a direct proportionality should result and the slopes of the regression lines for solute and solvent should be inversely proportional to the free diffusion coefficients of solute and solvent. The observed flux ratio for water as well as the theoretical flux ratio for urea is also shown in Fig. 1. The shaded area includes $\pm$ two standard errors of the mean about the regression line for urea which was calculated by the method of least squares. Although there is a considerable scatter in the data, they conform sufficiently with theory to indicate a definite interaction between the movement of urea and that of water through the bladder consistent with net transfer of water by bulk flow and utilization of common pathways for penetration by urea and water.

Reflection Coefficients for Penetration of Bladder by Solvents

Staverman (7) has indicated that penetration of membranes by solute relative to water can be simply expressed by the reflection coefficient, σ. The reflec-

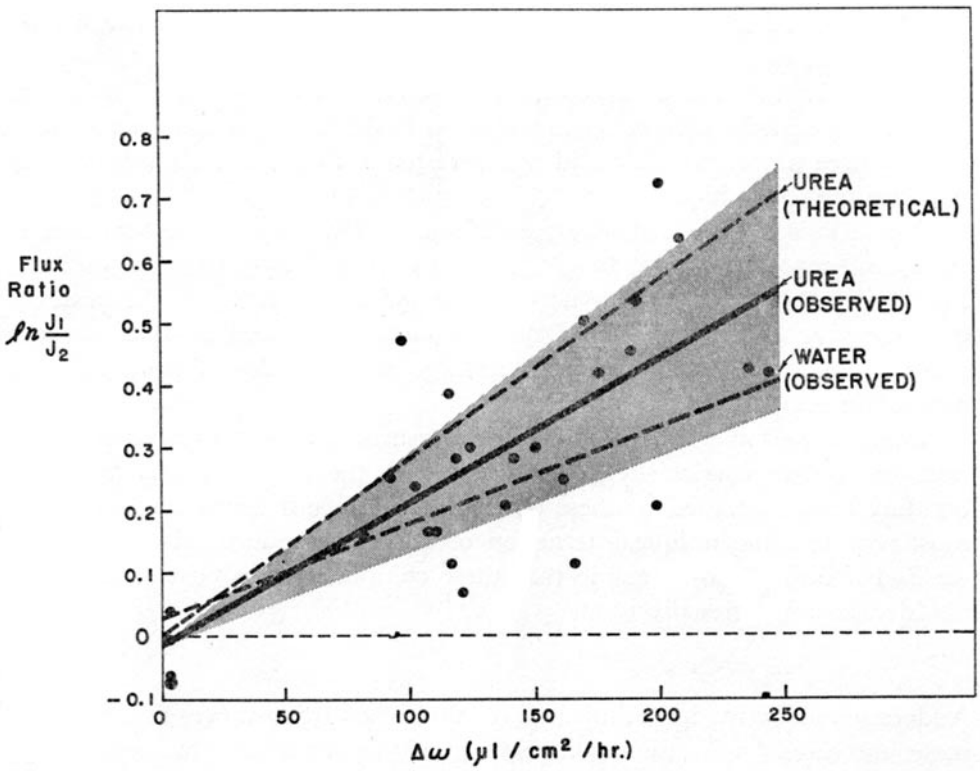


FIGURE 1. Effect of net water movement (ΔW) on the flux ratio of urea. C^{14}-labeled urea was used to measure unidirectional flux, J_1, and N^{15}-labeled urea to measure simultaneously the opposing unidirectional flux, J_2. Net movements of water were induced by osmotic gradient across the bladder wall in the presence of vasopressin. In the absence of net movements of water the unidirectional fluxes for water and urea, respectively, were equal in the two directions across the bladder as indicated by intersection with the ordinate at the origin. The equations for the lines (calculated by the method of least squares with standard errors for slopes and intercepts (8)), which best represent the observations, are for water and urea:

$$y = (0.0016 \pm 0.00015)\ x + (0.028 \pm 0.011)$$
$$y = (0.0023 \pm 0.0004)\ x - (0.0194 \pm 0.025)$$

respectively. The theoretical regression for urea is also indicated, see text for explanation. The shaded area includes twice the standard error of the slope for the urea regression. Although there is considerable scatter of the experimental values a definite interaction between solvent and solute fluxes is seen which supports the view that urea and water traverse the bladder through the same channels.

tion coefficient is calculated as the ratio of the concentration of the solute in the increment of water transported across the membrane to the concentration of the solute in the medium, subtracted from 1.0. Thus, a truly semipermeable membrane will act as a perfect sieve, the concentration of impermeant solute in the filtrate will be zero, and $\sigma = 1.0$. On the other hand, a solute which

penetrates the membrane without restriction will have the same concentration in the filtrate as in the medium and $\sigma = 0$.

Table IV shows the reflection coefficients obtained for thiourea and chloride and calculated for urea from the flux ratios shown in Fig. 1. With C^{14}-thiourea or Cl^{36} added to a dilute mucosal bathing medium the transmembrane permeability coefficient was measured first in the absence of net water movement and again with large net water transfer induced by addition of vasopressin. The increment in thiourea or chloride permeability and the measured net water transfer after addition of vasopressin were used to calculate σ. The data show that even in the presence of large net movements of water the membrane retains a high degree of impermeability to thiourea and chloride

TABLE IV

REFLECTION COEFFICIENTS FOR THIOUREA, CHLORIDE, AND UREA THROUGH TOAD BLADDER

	No. of experiments	Mean Δ_w	Mean σ	Range σ
		$\mu l/cm^2/hr.$		
Thiourea	6	200	0.995	0.988–1.00
Chloride	3	207	0.993	0.990–0.994
Urea	29	200	0.79	

$$\text{Reflection coefficient, } \sigma = 1 - \frac{\dfrac{\text{Net solute flux}}{\text{Net water flux}}}{\text{Medium concentration}}$$

while urea penetrates more readily. It is evident, however, that even urea suffers nearly an 80 per cent reduction in its rate of penetration relative to that of water.

Site of Selective Barrier to Penetration of Bladder by Solutes

With labeled urea or water in the mucosal bathing medium addition of vasopressin was found to increase significantly the tissue content of the labeled compound (4, 1). This established an action of vasopressin in or near the limiting plasma membrane lining the mucosal surface of the bladder. In order to determine whether the barrier to thiourea and other solutes whose penetration is not accelerated by vasopressin is in the same or opposite surface of the mucosal cells, similar measurements were made. The results in seven experiments on paired half-bladders indicate that in the absence of vasopressin the concentration of labeled thiourea in tissue water expressed as per cent of its concentration in the mucosal bathing medium is very low averaging 2.6 per cent and shows no significant increase with vasopressin, averaging 3.5 per cent in the paired half-bladders exposed to this hormone. The standard

error of the mean difference of +0.9 is ±0.4 per cent. The values of 2.6 and 3.5 per cent without and with vasopressin for thiourea are to be contrasted with the corresponding mean values of 11 and 38 per cent for urea (4). The low values for thiourea as compared with those for urea indicate that the mucosal surface of the bladder constitutes the major permeability barrier to thiourea and this barrier is unaffected by vasopressin in the case of thiourea.

DISCUSSION

Koefoed-Johnsen and Ussing (2) deduced from the disparity between observed net water transfer and diffusional net water transfer across the toad skin that bulk flow of water through pores must be occurring. They ascribe the increased net water transfer induced by antidiuretic hormone to an action of the hormone to enlarge the size of such aqueous channels. From a study of the effects of solvent drag on the movement of thiourea and acetamide through toad skin Andersen and Ussing (6) obtained further support for their thesis.

We have also shown for the toad bladder (1) that the net flux of water contributed by diffusion is inadequate to account for the observed large net transfers of water which occur in the presence of vasopressin. Considerations noted above regarding the permeability coefficients of the compounds listed in Tables I and II also support the view that the hormone modifies aqueous channels in the membrane. Likewise the solvent drag studies on urea reinforce the view of the earlier workers that this non-diffusional transport of water occurs in fact by bulk movement.

In contrast, however, to such apparent evidence supporting the existence of sizeable aqueous channels through the membrane, and in keeping with the needs of the animal to maintain selectivity in the reabsorption of small solutes from the bladder urine, the hormonal effects on solute penetration appear to be highly specific. The unidirectional permeability of the membrane to most small molecules and ions is very low and undetectably affected by vasopressin. This is true, as demonstrated for thiourea and chloride, even in the presence of larger net water transfers through the bladder.

On the other hand, the penetrability by a small group of molecules typified by the important urinary solute, urea, is markedly affected by hormone. The common feature of this group of compounds is the possession of an amide or amide-like group. These compounds appear to penetrate passively and no evidence for carrier-mediated transport has yet been elicited although some specific interaction between amide group and the membrane would appear likely. Until more is understood regarding the nature of this specificity, however, this group of compounds is considered to penetrate by diffusion and the specificity is ascribed to physical factors relating to the amide grouping or

to hydrogen bonding. Certainly molecular size alone cannot be the distinguishing feature as the dimensions of compounds in this group must overlay some in the group unaffected by hormone.

Thus, from estimations of non-diffusional transport of water and of solvent drag effects, a picture of the membrane as honeycombed with sizeable aqueous channels permitting bulk flow of water is derived which seems inconsistent with the specific hormonal effects on penetrability of the membrane by small molecules and the measured reflection coefficients of such small solvents. Andersen and Ussing (6) have suggested that selectivity may reside in a thin diffusion barrier which is in series with a porous barrier. The former would constitute the permeability barrier to substances in Table I while the latter would be the rate-limiting barrier for water and for those compounds in Table II. It would be this porous barrier which is affected by neurohypophyseal hormones. This concept is at present the most satisfactory explanation for the selective permeability characteristics noted.

The present results permit us to localize this hypothetical series barrier in the bladder. The thin selective diffusion barrier must be in or near the plasma membrane at the mucosal surface of the bladder to keep the tissue content of thiourea as its very low values. The porous barrier which is affected by vasopressin must also be at the mucosal surface to account for the increase in tissue labeling with urea (4) and water (1) as well as the bulk flow of water at this surface induced by the hormone (1). The plasma membrane at the serosal surface of the epithelial layer must include at least the selective diffusion barrier in order to preserve the cellular content of essential small solutes in the presence of large transcellular net movements of water. If a porous barrier creating transfer of water by bulk flow across the serosal surface of the mucosal cells is also present, the aqueous channels are unresponsive to vasopressin and fixed in the "open" state.

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