Some Actions of Neurohypophyseal Hormones on a Living Membrane

ALEXANDER LEAF

From the Departments of Medicine, Massachusetts General Hospital and the Harvard Medical School, Boston, and the Howard Hughes Medical Institute

It has long been recognized that the antidiuretic hormone of the neuro hypophysis must have an action on the renal tubule which results in enhanced reabsorption of water. Ussing and Zerahn (1) have demonstrated in amphibian membranes that this hormone also stimulates active sodium transport. Whether it may in addition influence the reabsorption of other substances, however, has not been fully investigated. I should like to report some recent investigations which indicate: (*a*) that the hormone has a specific effect with respect to another major urinary constituent, urea, (*b*) that the cellular site of hormonal action can be localized, and (*c*) that a simple unitary hypothesis reasonably explains, at the present time, the observed actions of the hormone.

Although our interest in this subject began with the mammalian kidney, the difficulties inherent in studying that complex organ have led us to investigate an organ which is anatomically simpler, but which mimics the activities of the mammalian renal tubule in several important respects. Fig. 1 shows a histological section of the urinary bladder of the toad, *Bufo marinus*. The bladder is a bilobed structure which, in the intact animal, may occupy as much as one-half the abdominal cavity. Occasionally one sees a bundle of smooth muscle or a capillary in the bladder wall but most of the tissue appears, like that shown in higher magnification to the right, as a single layer of mucosal cells supported on a minimum of connective tissue.

Electrical Activity and Active Sodium Transport

Despite its transparent thinness, a potential of some 10 to 110 mv. can regularly be measured across the membrane, with the mucosal, or urinary side, electronegative with respect to the serosal side. This electrical activity of the bladder can be entirely accounted for by an active transport of sodium from the mucosal to the serosal surface (2). This was demonstrated by application

The studies here reported were supported in part by grants from the John A. Hartford Foundation, Inc., the United States Public Health Service (Grant H2822), and the American Heart Association, Inc.

of the short-circuiting and double isotope labeling techniques of Ussing and Zerahn (1) to the bladder mounted as a membrane separating two halves of a lucite chamber. When both surfaces of the membrane are bathed with the same Ringer's solution and the spontaneous membrane potential is nullified

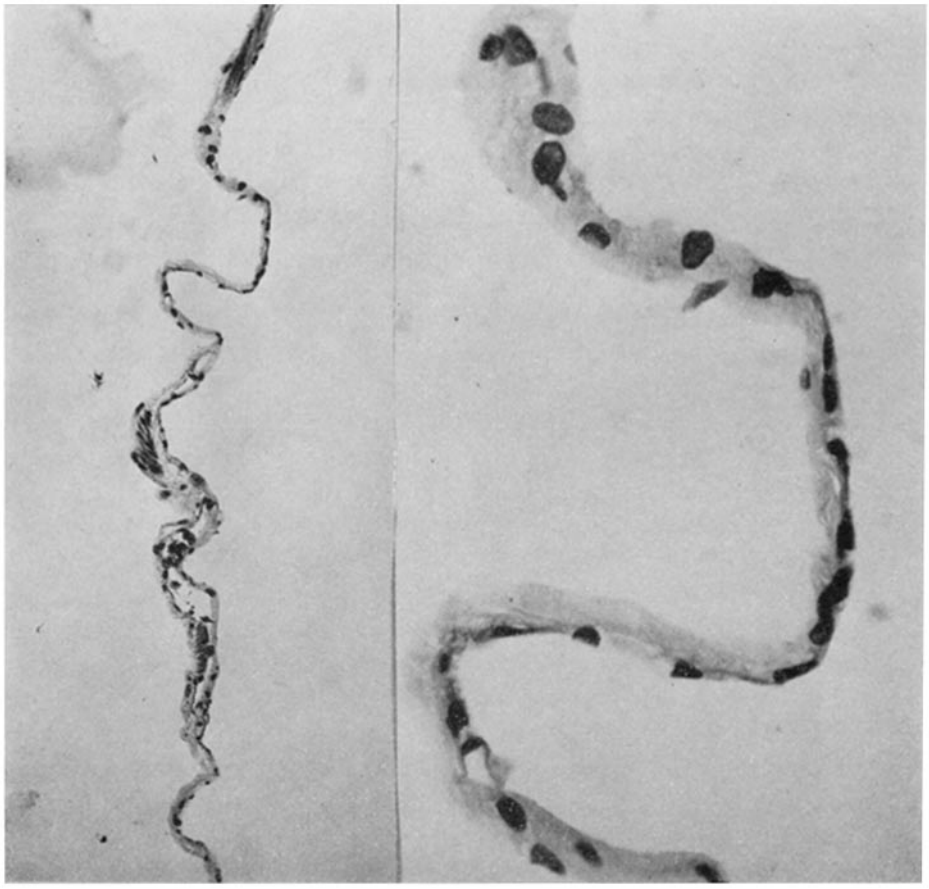

FIGURE 1. Cross-section of bladder wall seen under low magnification (× 125) on the left and with higher magnification (× 450) on the right. The mucosal or urinary surface faces the right and consists of a single layer of cells. These epithelial cells are supported on a minimum of connective tissue, as seen on the right. The surface opposite the mucosa has a serosal covering.

by an imposed external E.M.F., a current flows through the external circuit. To determine the source of this electric current double isotope labeling experiments with sodium were performed in the short-circuited preparation. Results like those shown in Table I were obtained. The radioactivity isotope, Na^{22}, was used to measure mucosal to serosal movement of sodium, and another isotope, Na^{24}, was employed to measure simultaneously the flux of sodium

from the serosal to mucosal surface. When the smaller serosal to mucosal flux is subtracted from the larger flux in the opposite direction, the mean net sodium flux in the direction of reabsorption from bladder urine is found to equal the simultaneously measured short-circuit current.

The expectation for the passive movement of ions in the absence of an electrochemical gradient is an equal flux in both directions. Therefore, demonstration of a unilateral net movement of sodium ions across the short-circuited bladder is proof that the membrane must be doing work to produce such asymmetrical movement of these ions. Equality of net sodium flux and of the short-circuit current indicates, furthermore, that the only ion involved in active transport across the bladder is the sodium ion. This demonstration of

TABLE I

COMPARISON OF THE NET SODIUM FLUX AND THE SHORT-CIRCUIT CURRENT THROUGH THE ISOLATED URINARY BLADDER OF THE TOAD, *BUFO MARINUS*

Sodium flux was measured from mucosal to serosal surfaces (M to S) by means of Na^{22} and simultaneously in the opposite direction (S to M) with Na^{24}. The short-circuit current was read at the same time directly from a micro-ammeter.

Periods		Mean Na flux		A	B
Number	Duration	M to S	S to M	Mean net Na transport	Mean short-circuit current
	min.	*μa./cm.²*		*μa./cm.²*	
16	60	57.5	15.3	42.2	43.1

Mean difference (A − B) = -0.9μa./cm.²
S.E. of mean difference = $\pm 2.9\mu$a./cm.²

active sodium transport by the isolated toad bladder is similar to that previously made for frog skin by Ussing and Zerahn (1).

Permeability Characteristics and Their Modification by Hormone

The permeability of the bladder to a given molecule or ion can be directly determined *in vitro* with the membrane separating two halves of a lucite chamber. A tracer amount of the substance of interest is added to the medium bathing one surface and its rate of appearance on the opposite side is measured. By this technique we have determined the permeability of the membrane to a considerable number of substances, most of which are summarized in Table II. Colloidal gold, 30 Å diameter particles, apparently failed to penetrate. A large number of substances penetrated at low rates of 1 to 20 $\times$ 10^{-7} cm./sec. and their rates of penetration were unaffected by neurohy-

pophyseal hormones. Urea has an initially low penetration but following addition of hormone[1] permeability to urea may increase as much as fortyfold. Sodium penetrability in the direction of active transport is uniformly increased and water permeability in the absence of an osmotic gradient is approximately doubled.

These results are summarized in Fig. 2 in which the abscissa indicates time, and on the ordinate is plotted the permeability coefficient, which is defined as the amount of a given molecule or ion crossing 1 cm.^{2} of membrane per second, under a driving force of unit concentration gradient. The solid

TABLE II

PERMEABILITY OF THE TOAD BLADDER WITHOUT AND WITH NEUROHYPOPHYSEAL HORMONES

Substance			K_{trans} ($\times 10^{-7}$ cm./sec.)	
			Without neurohypophyseal hormone	With neurohypophyseal hormone
Colloidal gold			0	0
Thiourea Glycerol Glycine Sucrose Inulin	Sodium (passive) Potassium Choline Chloride Iodide	Thiocyanate Sulfate Lactate Phosphate Arginine	1–20	1–20
Sodium (active)			35	100
Urea			26	274
Water			900	1600

lines indicate permeability in the direction mucosa to serosa, and the broken lines, permeability in the opposite direction.

The action of antidiuretic hormone has been classically considered to be that of increasing the renal tubular reabsorption of water. As indicated here, pitressin produces an approximately twofold increase in the permeability of the bladder to water. Double labeling experiments with D_2O and T_2O, in the absence of an osmotic gradient, have indicated that permeability to water is equal in both directions and hence presumably passive. Note that the per-

[1] "Hormone" will be used in this report to apply to mammalian neurohypophyseal hormones. In most instances commercial vasopressin has been employed. However, both purified arginine-vasopressin and oxytocin, generously supplied by Professor V. du Vigneaud, have been tested in the applications referred to and have yielded identical results. Hormone was usually added to yield a concentration in the medium of 100 milliunits per ml. but the preparation responds similarly to much lower concentrations.

meability coefficient for water is charted on a separate scale on the right, and that its absolute magnitude is considerably greater than that of the other substances shown.

In addition to this effect of the hormone on the permeability to water, a marked effect has also been elicited with respect to the permeability of the membrane to urea, as shown by the top lines in Fig. 2. After addition of pitressin, the low initial permeability to urea is increased as much as forty-

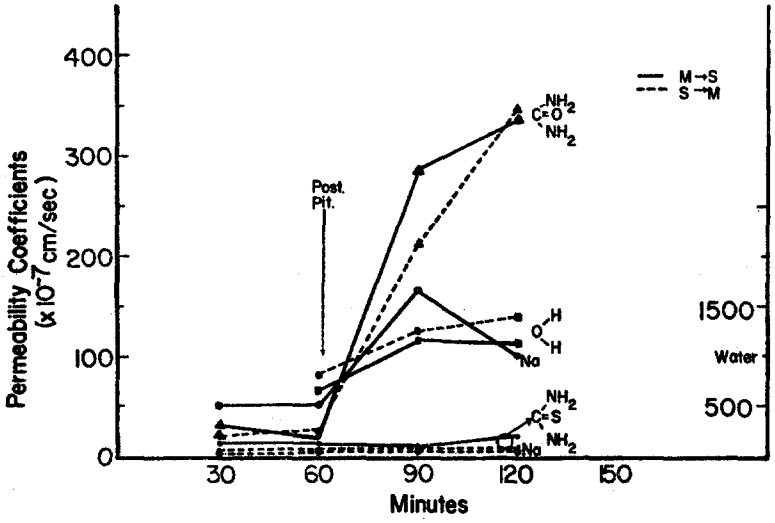

FIGURE 2. Permeability of toad bladder *in vitro* to water, urea, sodium, and thiourea. The ordinate is the permeability coefficient, and the abscissa, time. Results of representative experiments are shown. After one or two 30 minute control periods posterior pituitary extract was added to the bathing medium, and permeability measurements continued for an additional two 30 minute periods. Note that in the absence of an osmotic gradient permeability to water was doubled and similarly affected in both directions. Urea permeability also increased greatly in both directions while only active sodium transport was stimulated. No change in permeability to thiourea occurred. M → S designates unidirectional flux from mucosal to serosal surfaces, and S → M the flux in the opposite direction.

fold, and, as for water, the effect has been found to be the same in the two directions (3). This was demonstrated in double labeling experiments in which C^{14}- and N^{15}-labeled urea were used to measure simultaneously the permeability of the membrane to urea in the two directions. The good agreement for permeability coefficients both before and after hormone excludes the presence of an active transport process. When non-labeled urea was added to the bathing medium in concentrations up to 50 mM no self-depression of the permeability of the labeled urea was noted, suggesting that the high permeability after hormone is not a carrier-mediated process; its mode of passage would appear to be by free diffusion.

Although the action of the hormone in enhancing the movement of water and urea through the membrane is a passive phenomenon, and could be attributed to a simple dilatation of aqueous channels, the permeability characteristics of the membrane, nonetheless, retain a high degree of specificity. This is strikingly shown by the complete absence of an effect on the permeability to thiourea, shown in the lower part of Fig. 2. Thiourea is a small molecule whose chemical formula can be seen to resemble quite closely that of urea.

Recently we have examined acetamide and two of its analogues, proprionamide and butyramide. The permeability of the membrane to acetamide is also affected by hormone. With proprionamide and butyramide the pitressin effect is still definite, but with the longer chain molecules lipoid solubility increases and apparently with this the permeability of the membrane to these compounds increases even in the absence of the hormone. The results with these compounds suggest that ability to hydrogen-bond may be related to this pitressin effect. How the ability to hydrogen-bond can be related to an accelerated diffusion, however, escapes any ready explanation from me at present.

In contrast to these effects on passive movement, the hormone exerts a definite action on the active transport of sodium, seen in Fig. 2 as the upper solid sodium line. The higher permeability of the membrane to sodium, in the direction mucosa to serosa, that is in the direction of reabsorption from the urine, is attributable to the active transport of this ion as discussed. The hormone is seen to stimulate only the active sodium transport; passive movement, in the direction serosa to mucosa, is not affected.

Another interesting feature of this hormonal action which we will refer to again later is that it occurs only when the hormone is applied to one surface of the membrane (2). When hormone is added, even in large amounts, to the medium bathing the mucosal surface, no effect has been observed. The same amount of hormone added to the serosal bathing medium invariably produces a prompt increase in sodium transport and in the permeability to urea and water.

Energy Requirements for Transport

The active transport of sodium will occur up-hill, against an electrochemical activity gradient. This active process requires the expenditure of energy, energy which derives from the metabolism of the tissue. Accordingly, the enhanced sodium transport stimulated by posterior pituitary hormones should be accompanied by an increased rate of tissue metabolism. That this is indeed so is shown in Table III. The figures represent values for oxygen consumption expressed as QO_2; that is, microliters of oxygen consumed per

milligram of dry tissue in one hour. The top line indicates that there is no change in oxygen consumption between the 1st and 2nd hours in the control half-bladders. However, when hormone was added after one hour to the paired bladder half, a significant increase in oxygen consumption resulted. When comparable studies were repeated in sodium-free media, as shown below, the hormone had no demonstrable effect on oxygen utilization. Analogous results have been obtained with respect to glycolysis and lactate formation, as shown in Table IV. The glycogen content of the bladder half incubated aerobically without neurohypophyseal hormone is seen to be higher and the lactate production lower than that for the other half-bladder treated

TABLE III

EFFECT OF NEUROHYPOPHYSEAL HORMONES ON QO_2 OF TOAD BLADDER

Measurements of oxygen consumption were made for 3 consecutive hours on paired bladder halves in Warburg vessels by classical manometric techniques. Hormone was added at the end of one hour to one bladder half while the other served as control. Measurements were made in sodium-Ringer solution (A) and in sodium-free Ringer solution (B) in which all the sodium had been replaced by magnesium or choline (2).

	Hours			Mean difference (hours 2 − 1)	S.E. mean difference	*P*
	1	2	3			
A. Sodium-Ringer	(10 paired experiments)					
Control	1.20	1.22	1.06	+0.02	±0.03	0.6
With hormone*	1.23	1.72	1.76	+0.49	±0.05	<0.001
B. Sodium-free Ringer	(19 paired experiments)					
Control	1.01	0.97	0.93	−0.04		
With hormone*	1.12	1.12	1.10	0		

* Hormone added at end of first hour.

with hormone. In the absence of sodium, however, these effects of hormone on glycolysis and lactate production disappear. From these results it is apparent that stimulation of sodium transport by neurohypophyseal hormone is associated with an increase in the energy metabolism of the tissue; on the other hand, in the absence of sodium the hormone exerts no detectable effect upon energy metabolism.

Although this action on sodium transport is definitely associated with an increase in the energy metabolism of the tissue, the hormonal action with respect to urea and water is not energy-dependent. Enhanced urea and water permeability with hormone have been demonstrated in the complete absence of sodium, under which circumstances, as just seen, there is no discernible effect on energy metabolism. This observation is in keeping with the passive nature of the passage of water and of urea through the membrane.

Site of Hormonal Action

Thus far, we have demonstrated that the actions of the neurohypophyseal hormones affect the passive permeability of the membrane to urea and water and stimulate the active transport of sodium. I should like next to indicate the site in the membrane at which the hormone acts. In order to explain the rationale for the experiments used here a brief digression to fill in some background is necessary.

During the course of studies on the anaerobic metabolism of this tissue it was regularly observed that more lactate accumulated in the medium bath-

TABLE IV

TISSUE GLYCOGEN CONTENT AND LACTATE FORMATION BY TOAD BLADDER AEROBICALLY WITH AND WITHOUT NEUROHYPOPHYSEAL HORMONES

Glycogen content of tissue and lactate content of bathing media were measured on paired bladder halves to one of which hormone was added. Time of incubation was 45 minutes and equal for both bladder halves.

Glycogen content			Lactate formation		
Control	Hormone	Δ	Control	Hormone	Δ
μg./mg. tissue			*μg./mg. tissue*		
A. Sodium-Ringer solution (8 paired experiments)					
2.13	1.73	−0.40	0.22	0.52	+0.31
S.E. Mean difference		±0.077			±0.036
P		<0.001			<0.01
B. Sodium-free Ringer Solution (8 paired experiments)					
2.82	2.62	−0.19	0.22	0.24	+0.022
S.E. Mean difference		±0.16			±0.032
P		0.3			0.5

ing the serosal surface of the membrane than in that bathing the mucosal side (4). As the membrane is symmetrical with respect to both the composition of the medium bathing its two surfaces and its electrical properties (measurements made on short-circuited membranes), this finding worried us until we realized that the lactate formed, presumably within the mucosal cells, might encounter a high resistance to outward diffusion at one cell surface and a lower resistance at the other. More lactate would of course appear in the medium bathing the latter side than in that bathing the former. Such a situation is schematically indicated in Fig. 3. It was assumed that lactate formed within the membrane must encounter at least two diffusion barriers to its exit at the opposite surfaces of the membrane. When the relative permeability coefficients, k_1 and k_2 of these two diffusion barriers, were actually determined (5), k_1 was found to average 5.4×10^{-7} cm./sec. while k_2 averaged

75×10^{-7} cm./sec. Thus the permeability of the serosal surface is some fifteen-fold greater to the passage outward of lactate from cell to medium than is the mucosal surface. Furthermore, k_1 was found to be equal when measured separately in the two directions across the membrane, and so was k_2. An equal permeability coefficient in the two directions across a membrane is consistent with a passive process of penetration. Thus we can conclude that lactate passes through the membrane passively but that it encounters a much higher barrier to diffusion through its mucosal surface than through its serosal surface. When one considers the simple histology of this membrane one per-

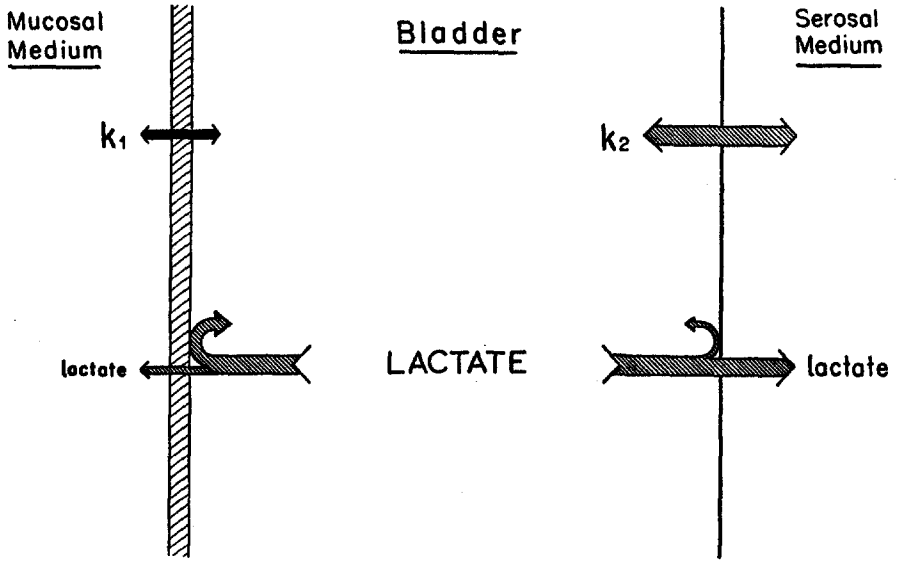

FIGURE 3. Schematic diagram of the barriers to diffusion encountered by lactate formed within the bladder to passage into the bathing medium. The mucosal surface proves to be much less permeable to lactate than the serosal surface. k_1 and k_2 are the relative permeability coefficients of these surfaces, respectively.

haps has justification for identifying these two diffusion barriers with the opposite surfaces of the single layer of mucosal cells. Fortunately for such an assumption the high resistance or low permeability surface is the bare mucosal cell surface while the high permeability surface includes the connective tissue and serosa. These studies with lactate permeability constitute our justification for dealing with the membrane as though it had two diffusion barriers.

This analogy may be now extended a step further to localize the site of hormonal action in the membrane. Realizing that the effect of the posterior pituitary hormones is to increase the transmembrane permeability to both urea and water, it will be evident that hormone must affect the permeability barriers in the membrane. Reference to the diagrammatic sketch in Fig. 4 will show a simple method to determine at which barrier the hormone must act.

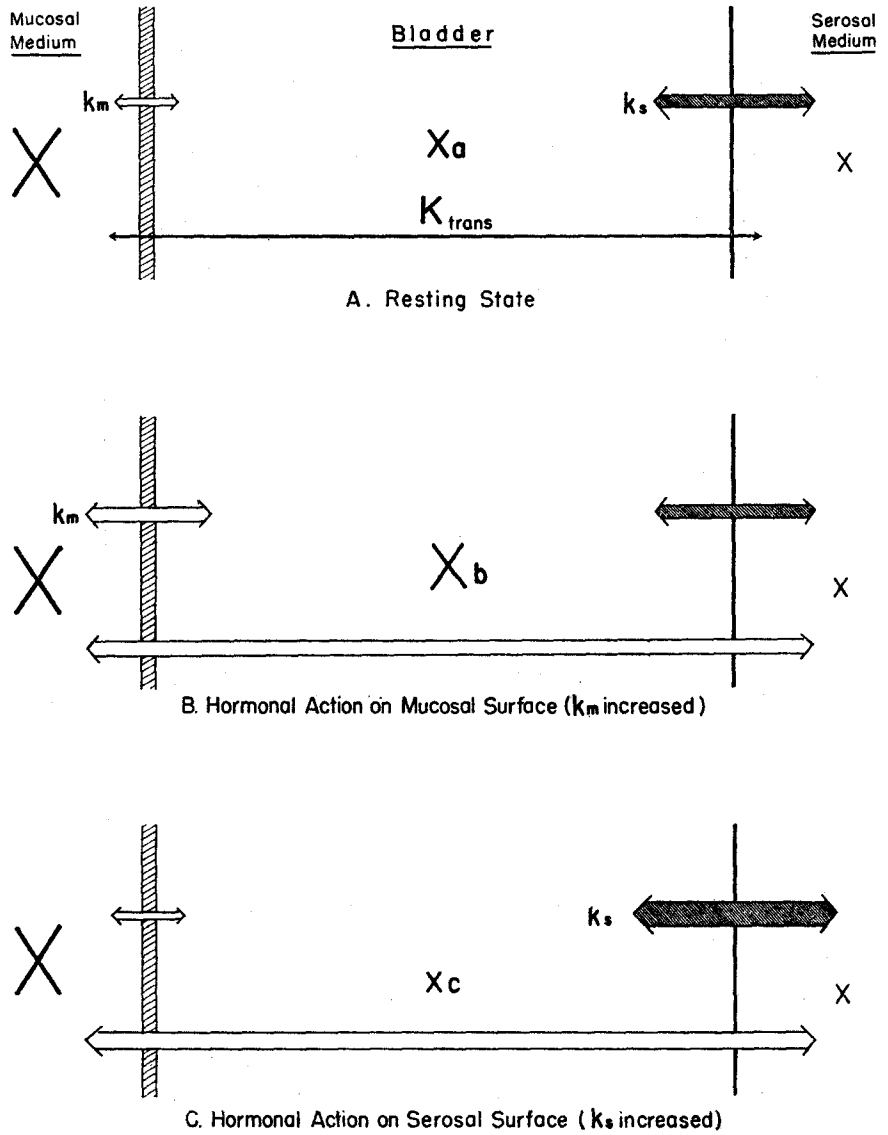

FIGURE 4. Schematic diagram to illustrate the method of determining at which surface of the membrane the hormone acts. Labeled water or urea in these experiments was initially added to the mucosal bathing medium to yield a concentration of tracer, X, in the medium. A represents the resting, non-hormone-treated state. B shows the increase in the transmembrane permeability coefficient, K_{trans}, and of the tissue concentration, X_b, that would result from an action of the hormone to increase the permeability of the mucosal diffusion barrier, k_1. A similar increase in K_{trans} might be achieved by an action of the hormone to increase the serosal permeability, k_2, as shown in C, but such an effect would result in a drop in tissue concentration, X_c, of the labeled molecule. Hence the criterion for a mucosal site of action of hormone is that $X_b > X_a$ and for a serosal site of action that $X_a > X_c$.

Labeled urea or water is added to the mucosal bathing medium, and its concentration in tissue water determined following a given period of incubation. If the hormone acts to increase k_1 the result will be an increase of the concentration of labeled urea and water in the tissue following hormone, while if the action is to increase k_2 the concentration of these two substances should decrease in the tissue after hormone. Thus the concentration in the tissue of labeled urea or water placed initially in the medium bathing the mucosal surface will be greater than in the non-hormone-treated control (Fig. 4A) if the site of action is on the mucosal diffusion barrier (Fig. 4B). If the action is on the serosal diffusion barrier the tissue concentration of these two tagged substances will be lower than in the control (Fig. 4C).

TABLE V

THE EFFECT OF NEUROHYPOPHYSEAL HORMONES ON THE LABELING OF TISSUE WATER BY C^{14}-UREA PLACED IN MUCOSAL MEDIUM

	Without hormone	With hormone	Time elapsed after C^{14}-urea added
	*per cent labeling**		*min.*
Paired bladder halves	6	10	8
	6	17	9
	5	46	60
	15	30	123
	13	45	153

* Per cent labeling = $\frac{\text{Average concentration in tissue water}}{\text{Concentration in mucosal medium}} \times 100.$

Table V compares the concentration of C^{14}-labeled urea in tissue water in paired bladder halves, one of which was treated with hormone. The labeled urea was added to the mucosal bathing medium and the time of exposure to the labeled medium of each bladder pair was identical. The concentrations in tissue water are expressed as per cent labeling, which is the average concentration of labeled urea in the tissue water divided by its concentration in the medium. In each instance the concentration in tissue water was increased in the tissue treated with hormone. A larger group of experiments (3) confirms the few results seen here. Table VI shows that similar results are obtainable with labeled water; a higher concentration of label is found in the bladder half treated with hormone. These findings clearly demonstrate that, with respect to water and urea permeation, the action of neurohypophyseal hormones must be to increase the permeability, at least, of the diffusion barrier located at or near the mucosal surface of the single layer of epithelial cells in this tissue. The data do not allow us to exclude an additional minor action on the serosal diffusion barrier, but we can say that

the major action of the hormone on urea and water permeability must result from its action on the mucosal surface.

This localization of the action of the hormone is all the more striking when one recalls that the hormone produces effects only when added to the medium bathing the serosal surface. When added to the mucosal bathing medium it exhibits no effects. Hence its site of action is on the surface opposite from that on which it must be applied. This may represent another manifestation of

TABLE VI

THE EFFECT OF NEUROHYPOPHYSEAL HORMONES ON THE LABELING OF TISSUE WATER BY TRITIATED WATER PLACED IN MUCOSAL MEDIUM

Number of paired experiments	Without hormone	With hormone
	*per cent labeling**	
10	20.8	27.2
	$\Delta = 6.4 \pm 2.3 \quad (P = 0.02)$	

* Per cent labeling $= \frac{\text{Average concentration of } T_2O \text{ in tissue water}}{\text{Concentration of } T_2O \text{ in mucosal medium}} \times 100.$

the marked permeability barrier at the mucosal surface which may even prevent the purified hormone from gaining access to its site of action from that surface.

An Hypothesis of Hormonal Action

Fig. 5 summarizes our current hypothesis concerning the actions of the neurohypophyseal hormones on the toad bladder. Here we have schematically depicted the membrane before and after the addition of hormone. The mucosal and serosal surfaces of the membrane are indicated, and the permeability to urea, water, and sodium is shown. The k values are representative permeability coefficients in the absence and presence of hormone. As described, both urea and water cross the membrane passively in both directions. Hormone increases this passive permeability by reducing the mucosal diffusion barrier.

Because it is actively transported, the movement of sodium across the membrane must be considerably more complex. From a consideration of tissue sodium concentration and of transmembrane electrical potentials it seems necessary to place the site of active transport at the serosal surface of the cell. The entry of sodium into the cell may well be a passive process, with diffusion down its concentration gradient; its removal from the cell, however, against both a concentration and an electrical gradient must require expenditure of energy. We have shown that the action of the hormone with respect

to sodium is to increase its movement across the membrane and to stimulate tissue metabolism. Can these hormonal actions on active transport be reconciled with the effects on the passive permeability to water and urea? At the present time, it would appear that the simplest unitary hypothesis is that the hormone increases the passive permeability of the mucosal barrier to urea,

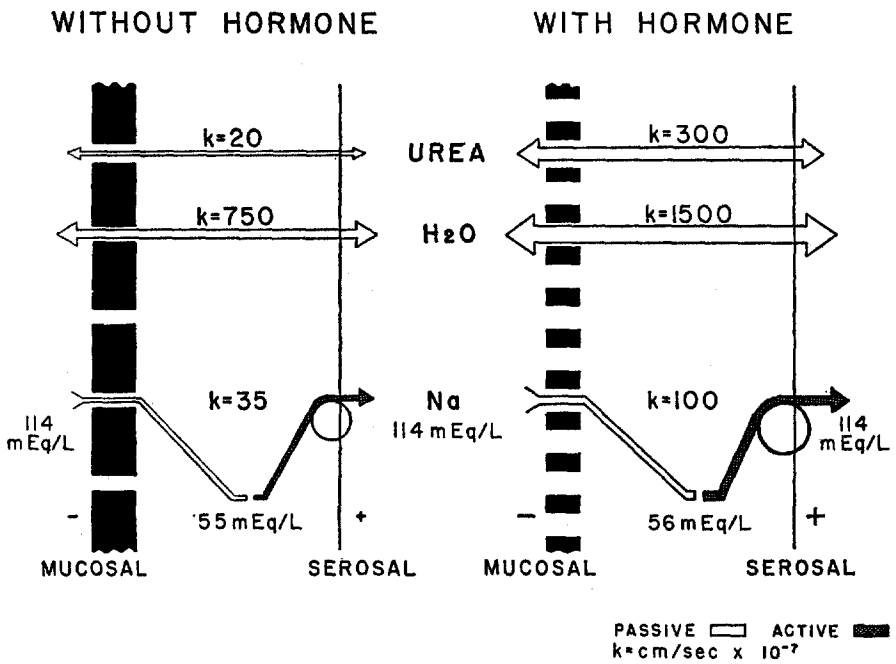

FIGURE 5. Schematic drawing to illustrate action of hormone to increase passive diffusion of water and urea and the active transport of sodium across the toad bladder. The effect of hormone is shown to be the result of an increased porosity of the mucosal surface. In the case of active sodium transport, an increased permeability of the mucosal surface, also resulting from hormone, is thought to accelerate the entrance of sodium into the cells from this side, and such entry may also be passive. Secondary to the increased entrance of sodium, an active transport process at the serosal side of the cells increases the rate of extrusion of sodium from the cell. Only the increased mucosal permeability is regarded as a direct effect of hormone.

water, and sodium. In the case of sodium, according to this hypothesis, such an increased rate of entry into the cells would result in a secondary increase of active sodium transport and hence of tissue metabolism. In this manner all the effects of the hormone could be ascribed to a single action at a single site in the cell.

When studies were carried out to demonstrate an action of the hormone on the mucosal diffusion barrier with respect to sodium, by the techniques used for urea and water, similar increases in tissue labeling by isotopic sodium were

noted, although no increase in chemically determined total intracellular sodium content has yet been found following hormone. The problem of establishing an unequivocal change in membrane permeability to an ion is much more difficult than in the case of uncharged molecules such as urea and water.

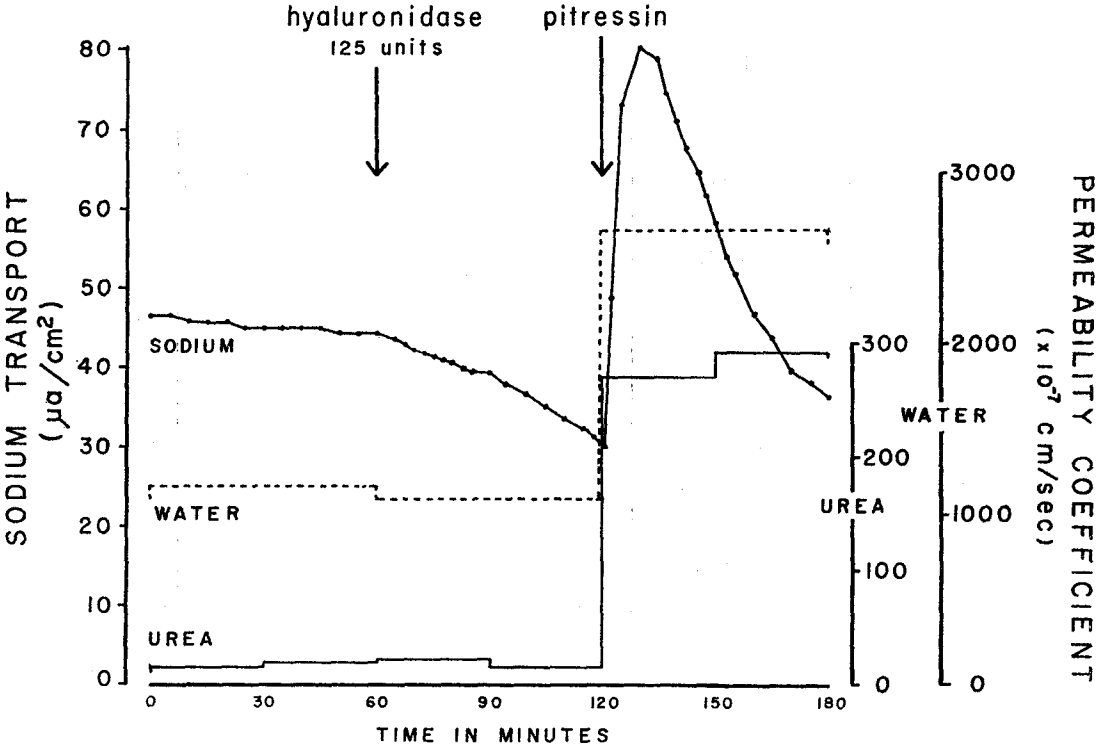

FIGURE 6. Results of experiments to test whether the specific actions on the toad bladder of mammalian neurohypophyseal hormones can be reproduced by hyaluronidase (wydase, Wyeth Laboratories, Philadelphia). As shown, hyaluronidase had no effect on water or urea permeability or active sodium transport even when large amounts were added to the bathing medium on both sides of the membrane. Posterior pituitary extract subsequently added still produced its large and specific effects on the permeability to these substances. Thus it seems unlikely that antidiuretic hormone acts through an effect of hyaluronidase.

Changes in the electrical potential across the diffusion boundary as well as in the permeability of the barrier will affect the rate of penetration by an ion. We cannot as yet, therefore, claim to have demonstrated rigorously an effect of the hormone on the mucosal permeability to sodium.

SUMMARY

Mammalian neurohypophyseal hormones have been shown to increase the passive permeability of the isolated toad bladder specifically to water and

urea and to stimulate active sodium transport through this membrane. The simplest hypothesis for explaining the observed effects of these hormones on the penetration of urea, water, and sodium through the bladder would be a selective action of the hormones to increase the permeability of the mucosal diffusion barrier to these substances. Such an effect has been definitely demonstrated for urea and for water and is strongly suggested for sodium.

Addendum Recently Ginetzinsky (6) has proposed that the action of the antidiuretic hormone is to cause an apocrine secretion of hyaluronidase by the renal tubules. The released enzyme was then thought to hydrolyze the hyaluronic acid on the mucosal surface of the tubular cells, thus rendering the tubular epithelium more permeable for the back diffusion of water. This interesting suggestion was based on his finding of an increased concentration of hyaluronidase in the urine during antidiuresis.

If this proposal were correct one might expect that hyaluronidase applied directly to the surface of the urinary bladder of the toad should reproduce the specific actions of mammalian antidiuretic hormone on this tissue. Fig. 6 shows that when commercial hyaluronidase, even in large amounts, was added to the medium bathing both surfaces of the isolated toad bladder it produced no detectable effect on permeability to water and urea or on active sodium transport. Subsequent addition of pitressin, however, still reproduced its characteristic effects. These findings make us suspect that the effects of the hormone are not mediated through an action of hyaluronidase.

This report includes results obtained during the past 3 years. It is a pleasure to acknowledge the collaboration of Dr. John Anderson, Dr. Howard S. Frazier, Dr. Richard M. Hays, Dr. Ezra Lamdin, Dr. Roy H. Maffly, and Dr. Lot B. Page, whose thoughts and efforts contributed much to the material included.

BIBLIOGRAPHY

1. USSING, H. H., and ZERAHN, K., *Acta physiol. scand.*, 1951, **23**, 110.
2. LEAF, A., ANDERSON, J., and PAGE, L. B., *J. Gen. Physiol.*, 1958, **41**, 657.
3. MAFFLY, R. H., HAYS, R. M., LAMDIN, E., and LEAF, A., *J. Clin. Invest.*, 1960, **39**, 630.
4. LEAF, A., PAGE, L. B., and ANDERSON, J., *J. Biol. Chem.*, 1959, **234**, 1625.
5. LEAF, A., *J. Cell. and Comp. Physiol.*, 1959, **54**, 103.
6. GINETZINSKY, A. G., *Nature*, 1958, **182**, 1218.