tral noradrenergic nerve endings (*16*).

Finally, some therapeutic implications of our findings may be noted. The general aim would be to correct or compensate for the presumed pathology of noradrenergic systems. One could try to increase the formation of DBH, or to promote supersensitivity of noradrenergic receptors (*5*), or even, ideally, to stimulate the regeneration of damaged noradrenergic terminals as, for example, by suitable central administration of nerve growth factor (*17*). Alternatively, in analogy to the use of L-dopa to elevate the central levels of dopamine in Parkinson's disease (*18*), one could try to increase the concentration of norepinephrine in the brains of schizophrenic patients by administration of a precursor (*19*).

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20. We thank J. Axelrod and R. Weinshilboum for guidance in setting up the DBH assay and M. M. Baden and M. H. Book for advice in selecting and obtaining human brain specimens. We also thank D. J. Reis for providing samples of phenylethanolamine-*N*-methyltransferase and A. T. Shropshire, W. J. Carmint, N. S. Buonato, and B. A. Brehmeyer for technical assistance.

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Vasopressin: Possible Role of Microtubules and Microfilaments in Its Action

Abstract. *Colchicine, vinblastine, podophyllotoxin, and cytochalasin B inhibit the action of vasopressin and cyclic adenosine monophosphate on osmotic water movement across the toad bladder. The findings suggest that microtubules, and possibly microfilaments, play a role in the action of vasopressin, perhaps through involvement in the mechanism of release of secretory material from the bladder epithelial cells.*

Vasopressin (antidiuretic hormone) stimulates contraction of certain types of smooth muscle and promotes transcellular water movement—and in some instances sodium transport—across the epithelium of the distal portion of the mammalian renal tubule, and amphibian skin and bladder (*1*). Vasopressin appears to promote water movement across responsive epithelial cells by inducing an increase in the permeability of the membrane at the apical surface of these cells (*2*); this action of the hormone is evidently mediated by cyclic adenosine monophosphate (AMP) (*3*). However, the molecular mechanisms involved in the permeability change induced by vasopressin and cyclic AMP are unknown.

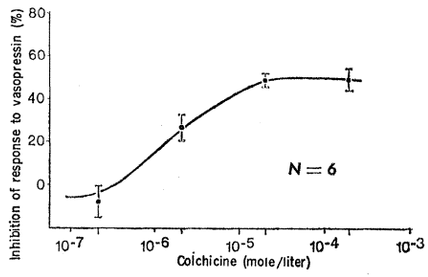

Fig. 1. The inhibitory effect of colchicine on osmotic water movement in response to vasopressin : dose-response curve. Colchicine was added 4 hours before the addition of vasopressin (20 munit/ml). Results are expressed as the percentage of reduction in the response to vasopressin in the colchicine-treated hemibladders relative to their paired controls, measured over a 2-hour period; each point represents the mean ± standard error of six to seven paired experiments.

The idea that an effect on common or analogous mechanisms may underlie the actions of vasopressin on smooth muscle and on epithelial tissues is appealing. Recent studies have suggested that microtubules and microfilaments are associated with several types of intracellular movement (*4, 5*), including chromosome movement (*6, 7*), the transport and release of secretory products (*8–10*), and cytoplasmic streaming (*4, 11, 12*); it has been postulated that these organelles participate in contractile mechanisms analogous to those occurring in muscle (*5, 7, 8, 11, 12*). We have therefore investigated the possibility that the action of vasopressin on the transcellular movement of water and sodium in epithelial tissues is mediated by a mechanism involving microtubules or microfilaments (or both).

The antimitotic agents colchicine, vinblastine, and podophyllotoxin, which exert disruptive effects on microtubules in vivo (*6, 13*) and interact with microtubule subunit protein in vitro (*14, 15*), have been used extensively as tools in the investigation of the role

of microtubules in cell function (*6, 8, 9*). Cytochalasin B (CB) has been reported to disrupt microfilaments in several cell types (*12, 16*) and has been shown by Spudich and Lin (*17*) to interact with actin. We have tested the effects of these agents on osmotic water movement and sodium transport in response to vasopressin and cyclic AMP in the isolated bladder of the Colombian toad *Bufo marinus*. Osmotic water movement across isolated bladders was measured gravimetrically (*18*). Paired hemibladders were mounted as bags (mucosal surface innermost), filled with 5 ml of water, or Ringer solution diluted 1:5, and suspended in 50 ml of aerated Ringer solution, consisting of (milliequivalents per liter) Na^+, 111.2; Cl^-, 113.0; K^+, 5.4; Ca^{2+}, 1.78; HPO_4^{2-}, 4.8; $H_2PO_4^-$, 0.6; *p*H 7.3; and (milliosmoles per kilogram) H_2O, 222. The test agents were added to the serosal bathing medium of one of each pair of hemibladders; 0 to 4 hours later vasopressin (20 munit/ml) or cyclic AMP (2 to 5 m*M*) was added to the serosal medium of both members of each pair. Water movement out of the paired hemibladders was measured by weighing each hemibladder bag at 30-minute intervals; weighing was continued for 2 hours after the addition of vasopressin or cyclic AMP. The sodium and potassium concentration of the fluid remaining in each bag at the end of the experimental period was determined by flame photometry. In the studies with CB, samples of the mucosal and serosal bathing media were removed for measurement of osmolality at 15-minute intervals. Sodium transport across freshly isolated hemibladders was measured in separate experiments by means of the short-circuit current technique (*19*). All studies were carried out at 24 ± 2°C. Values for *P* were calculated for paired data by means of Student's *t*-test. For electron microscopy, bladders were fixed in 1.0 percent glutaraldehyde and postfixed in 1 percent osmium tetroxide, followed by 0.5 percent uranyl acetate; thin sections were stained with uranyl acetate and lead citrate.

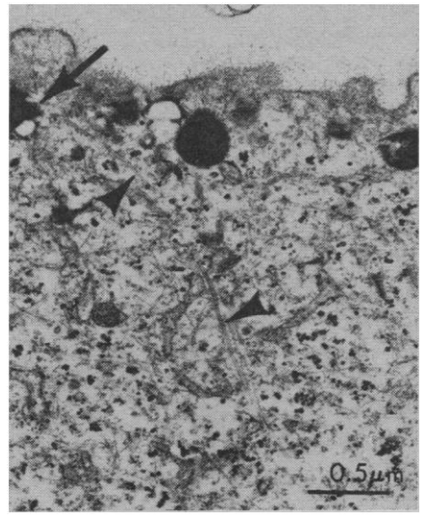

Fig. 2. Electron micrograph of granular epithelial cell of toad bladder mucosa. Note relationship of long microtubule (arrowhead) to secretory granule at apical surface of cell (arrowhead with stem).

In the absence of vasopressin, the rate of water movement across hemibladders exposed to an osmotic gradient was not influenced by colchicine ($2 \times 10^{-6}M$ to $2 \times 10^{-4}M$); for example, the mean weight loss of hemibladders exposed to $2 \times 10^{-5}M$ colchicine over a period of 4 hours was 0.7 ± 0.1 mg/min, and that of their paired controls was 0.8 ± 0.1 mg/min ($N = 13$), the difference being not significant. However, the rate of water movement in response to vasopressin was reduced in hemibladders previously exposed to these concentrations of colchicine (Table 1). The sodium and potassium content of the fluid within the hemibladders was not altered after exposure to colchicine; thus the reduced response to vasopressin was not due to a decrease in the osmotic gradient across the tissue. The dose-response relationship of the inhibitory effect of a 4-hour exposure to colchicine on the response to vasopressin is shown in Fig. 1.

The inhibitory effect of colchicine on the response to vasopressin was reversible—a characteristic of the effect of colchicine on microtubule systems in vivo (*6*): in hemibladders exposed to $2 \times 10^{-5}M$ colchicine for 3 hours and then transferred through four changes of fresh Ringer solution over the next hour, the rate of water movement following addition of vasopressin was comparable to that observed in control bladders.

The inhibitory effect of colchicine increased with the period of exposure up to 3 hours and leveled off thereafter. This time-dependence is similar to that reported for colchicine uptake by intact cells and for colchicine binding to microtubule protein in vitro (*15*). The similarity suggests that the time-dependence of the inhibitory effect on the response to vasopressin is not a function of cell permeability to colchicine, but rather that the observed effect is dependent on the binding of colchicine to microtubule protein.

Lumicolchicine, a structural isomer of colchicine, lacks antimitotic activity and does not bind to microtubule subunit protein (*15*). Exposure to $2 \times 10^{-5}M$ lumicolchicine (*20*) did not affect the rate of water loss from bladders exposed to an osmotic gradient in the absence of vasopressin nor did it affect the response to vasopressin (Table 1). The failure of lumicolchicine to interfere with the vasopressin response strongly suggests that the inhibitory effect of colchicine is specifically related to its ability to bind to microtubule subunit protein.

Cyclic AMP mimics the effects of vasopressin in the toad bladder; vasopressin apparently stimulates the synthesis of cyclic AMP in bladder tissue (*3*). The effect of cyclic AMP on osmotic water movement, like that of vasopressin, was markedly reduced in hemibladders which had been exposed to $2 \times 10^{-5}M$ colchicine (Table 1).

Colchicine ($2.5 \times 10^{-5}M$) had no effect on the baseline rate of active so-

Table 1. The effects of colchicine, lumicolchicine, vinblastine, and podophyllotoxin on osmotic water movement in response to vasopressin and cyclic AMP. All agents were added to the bathing medium of the experimental hemibladders 4 hours prior to the addition of vasopressin or cyclic AMP; n.s., not significant; S.E., standard error.

Test agent	*N*	Mean weight loss (mg/min)*		Percent inhibition† (mean ± S.E.)	*P*†
		Experimental	Control		
Vasopressin, 20 munit/ml					
$2 \times 10^{-5}M$ Colchicine	7	16.2 ± 2.3	30.7 ± 3.8	− 47.9 ± 3.0	< .001
$2 \times 10^{-5}M$ Lumicolchicine	6	37.2 ± 1.8	35.5 ± 3.1	+ 8.4 ± 9.8	n.s.
$2 \times 10^{-6}M$ Vinblastine	8	18.7 ± 0.9	36.2 ± 3.9	− 44.7 ± 5.4	< .001
$1.5 \times 10^{-5}M$ Podophyllotoxin	6	7.7 ± 1.7	30.1 ± 5.1	− 74.3 ± 3.7	< .001
Cyclic AMP, 2 mM					
$2 \times 10^{-5}M$ Colchicine	6	13.5 ± 3.9	35.8 ± 4.8	− 65.0 ± 5.8	< .001

* Measured over the 2-hour period after addition of vasopressin or cyclic AMP. † Calculated for paired experiments.

dium transport across the bladder. Furthermore, in contrast to its effect on vasopressin-induced water movement, prior exposure to $2.5 \times 10^{-5}M$ colchicine for 4 hours had no effect on the rise of sodium transport in response to vasopressin: following addition of vasopressin (20 munit/ml) the peak rise in the short-circuit current in the colchicine-treated hemibladders averaged 30 ± 5 μa, and that of their paired controls 23 ± 3 μa ($N = 12$), with no significant difference.

The effects of vinblastine and podophyllotoxin, in concentrations of $2 \times 10^{-7}M$ to $2 \times 10^{-5}M$, on osmotic water movement and sodium transport were similar to those of colchicine (Table 1). The observed dose-response relationships, reversibility, and time-dependence of the inhibitory effects of vinblastine and podophyllotoxin on vasopressin-induced water movement, like those of colchicine, are consistent with the characteristics of the action of these agents on other microtubule systems (*9, 13, 15*).

Exposure to CB (1 to 20 μg/ml) (*21*) caused a slight increase in the basal rate of osmotic water movement across the bladders. However, the rate of water movement following addition of vasopressin or cyclic AMP was reduced in a dose-dependent manner in bladders exposed to these concentrations of CB. Exposure to CB resulted in a leak of ions across the bladders from the serosal to the mucosal bathing medium, but the decrease in osmotic gradient across the tissue did not account for the reduced rate of water movement. Thus, after a 90-minute exposure to CB (5 μg/ml), water movement in response to vasopressin (calculated as milligrams per minute per unit of osmotic gradient) was reduced by 49.8 ± 6.1 percent ($N = 6$; $P < .001$). The inhibitory effect of CB was reversible: in hemibladders exposed to CB (5 μg/ml) and then transferred to fresh Ringer solution, the response to vasopressin equaled that of control bladders. CB had no effect on either basal or vasopressin-stimulated active sodium transport across the bladder.

Thus, colchicine, vinblastine, podophyllotoxin, and CB inhibit the action of vasopressin on osmotic water movement but do not affect the action of the hormone on sodium transport. The finding that these agents also inhibit action of cyclic AMP on water movement indicates that they exert their effects at sites distal to the action of vasopressin on cyclic AMP synthesis.

The disruptive effects of colchicine, vinblastine, and podophyllotoxin on microtubules in vivo (*6, 13*) and their direct interaction with microtubule subunit protein in vitro (*14, 15*) have been amply documented. These agents also have effects which do not appear to depend on an action on microtubules; thus colchicine and podophyllotoxin inhibit nucleoside uptake (*22*), and colchicine and vinblastine, in concentrations considerably higher than those used in our studies, have been reported to have an anticholinergic action (*22*) and to inhibit adenosine triphosphatase activity of brain actomyosin (*22*). The characteristics of the concentration- and time-dependence, and the specificity (as shown by the lack of effect of lumicolchicine), of the inhibitory effects of colchicine and other antimitotic agents observed in our studies are similar to the characteristics of their effects on microtubules in vivo (*6, 13*) and their interaction with microtubule protein in vitro (*15*); we therefore infer that the effects of these agents on vasopressin-induced water movement are due to their interaction with microtubule subunit protein. CB appears to disrupt actin-like microfilaments in many, but not all, cells (*12, 16*), but there is conflicting evidence concerning its ability to interact with actin in vitro (*17*). CB also inhibits hexose and nucleoside (*23*) uptake and these effects have been ascribed to interaction of the drug with plasma membranes (*23*). Thus while the inhibition of vasopressin-induced water movement by CB may be attributable to an interference with microfilament function or interaction with actin (or both), it is also consistent with an action of the drug on the plasma membrane of the bladder epithelial cells.

We conclude that the action of vasopressin on transcellular water movement in the toad bladder involves a mechanism in which microtubules—and possibly microfilaments—participate, whereas the action of the hormone on sodium transport does not involve these organelles. This conclusion is consistent with previous evidence that the effects of vasopressin on water and sodium movement are mediated by separate mechanisms—in particular with evidence that the effect of vasopressin on water movement is calcium-sensitive, while its effect on sodium transport is not (*24*).

DiBona *et al.* (*2*) have shown that the permeability change induced by vasopressin in the toad bladder is limited to the apical membrane of the granular epithelial cells. Masur *et al.* (*25*) have suggested that the permeability change is actually secondary to the release of secretion granules at the apical surface of the epithelial cells, and have obtained evidence that oxytocin and cyclic AMP stimulate the release by exocytosis of such granules from the granular cells (*25*). Mounting evidence indicates that the transport and release of secretory products in a variety of cell types is mediated by a mechanism involving microtubules (*8, 9*), perhaps microfilaments (*10*), calcium ions, and cyclic AMP (*26*); moreover, it has been postulated that this mechanism is contractile in nature (*8, 10*). In electron microscopic studies, we have in fact observed microtubules and conspicuous numbers of 100-Å microfilaments, apparently randomly oriented, in all four cell types present in the bladder epithelium. There is a well-defined microfilament network composed of 50- to 70-Å filaments in the microvilli and the subplasmalemmal region at the apical surface of the granular cells, and in some sections microtubules are seen in close relationship to one or more of the secretion granules lined up under the apical surface of these cells (Fig. 2).

On the basis of our findings we suggest that microtubules, and possibly microfilaments, may participate in the action of vasopressin on transcellular water movement in the toad bladder through their involvement in the mechanism of release of secretion granules from the bladder epithelial cells. We infer with Masur *et al.* (*25*) that the addition to the apical cell surface of secretory material and/or incorporation of the secretion granule membrane into the apical membrane of the granular cells may in turn be responsible for the change in membrane permeability. An alternative or even additional possibility is that microtubules and/or microfilaments play a role in the action of vasopressin through an effect on the rate of movement of water through the epithelial cell cytoplasm; for example, they might be involved in a directional cytoplasmic streaming process (*4, 11*).

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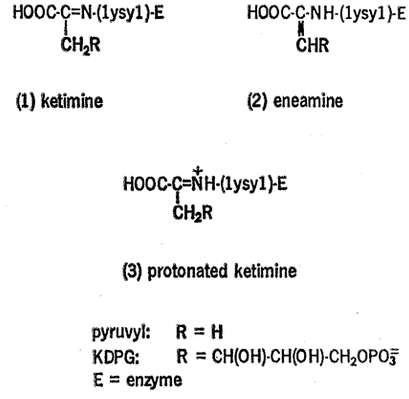
Aldolase Catalysis: Single Base–Mediated Proton Activation

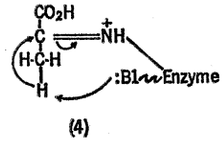
Abstract. *The enzyme, 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, catalyzes several reactions, the natural ones being (i) the exchange of hydrogen atoms of the methyl groups of pyruvate with protons of the solvent (C-H synthesis) and (ii) the reversible condensation of pyruvate with* D*-glyceraldehyde-3-phosphate (C-C synthesis). Previous work has provided chemical evidence for the occurrence of a protein-bound carboxylate group adjacent to the Schiff's base-forming lysine in the active site geometry. This carboxylate could provide the basic group postulated to participate in proton activation catalyzed by aldolases. With the use of three-dimensional models, it is shown that simple rotation about a carbon-carbon bond of the side chain will allow the base to assume the two positions necessary for proton activation in either the C-H synthesis or the C-C synthesis catalyzed by KDPG aldolase. This single base hypothesis provides a model wherein all reagents can approach a single face of the active site and is consistent with the stereochemistry thought to occur in the aldolase reaction.*

Aldolases have long been of great interest to biochemists because such enzymes provide a mechanism by which organisms can make and break carbon-carbon bonds. In this report we propose a model for the active site of 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, which catalyzes two mechanistically equivalent reactions: (i) the reversible condensation of pyruvate and D-glyceraldehyde-3-phosphate (G3P) to form KDPG and (ii) an exchange reaction between the methyl hydrogen atoms of pyruvate and protons of water (*1*). We show that it is possible for the base-assisted catalytic proton activation, thought to be required for the two substrates, to be accomplished by a single enzyme-bound carboxylate (instead of two different basic groups). This single base hypothesis provides a model wherein all reactants can approach a single face of the active site and is consistent with our present knowledge of the stereochemistry of aldolase reactions.

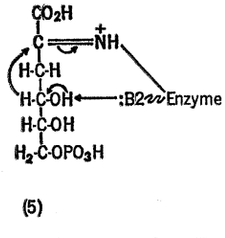
In 1964, Meloche and Wood (*1*) proposed a Schiff's base mechanism for KDPG aldolase. An amino group, provided by an enzyme-bound lysyl group, interacted with the carbonyl group of the substrate (pyruvate or KDPG). The intermediate Schiff's bases, specifically azomethines, represented by the tautomeric ketimine (**1**) and eneamine (**2**) forms, are shown below. A protonated ketimine (**3**) provided the electron sink necessary for catalysis.



In the presence of pyruvate the enzyme was inactivated by borohydride, since the intermediate lysylpyruvylazomethine (*1*, *2*) was reduced to form a secondary amine. Thus KDPG aldolase was considered a class 1 aldolase in the terminology of Rutter (*3*). It was suggested (*1*) that both pyruvate deprotonation and KDPG cleavage led to a common intermediate, a lysylpyruvyl eneamine (**2**) [analogous to the enolate formed when a metal-activated—that is, a class 2—aldolase, acts on its substrate (*3*, *4*)]. Specific proton activation for each substrate was considered (*3*–*5*) to be mediated by basic groups (visualized as B1 and B2 in **4** and **5**) in the active site of the enzyme. Such catalytic steps in KDPG



(4)



(5)

aldolase would cause for B1, deprotonation of the methyl group of pyruvate (**4**) thus leading to the formation of a lysylpyruvyl eneamine (**2**); and, for B2, deprotonation of the hydroxyl group at C-4 of KDPG (**5**) converting C-4 into a leaving group and allowing a rearrangement to G3P and the lysylpyruvyl eneamine (**2**).