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# Effect of Metabolic Inhibitors on Vasopressin-Stimulated Transport Systems in the Toad Bladder

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Vasopressin increases the permeability of receptor cells to water and, in tissues such as toad bladder, to solutes such as urea. While cyclic AMP appears to play a major role in mediating the effects of vasopressin, there is evidence that activation of the water permeability system and the urea permeability system involves separate pathways. In the present study, we have shown that inhibitors of oxidative metabolism (rotenone, dinitrophenol, and methylene blue) selectively inhibit either vasopressin-stimulated water flow or vasopressin-stimulated urea transport. There was no inhibition, however, when exogenous cyclic AMP was substituted for vasopressin, and little to no inhibition when the potent analogue 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) was employed. Rotenone had no effect on adenylate cyclase activity or cyclic AMP levels within the cell; dinitrophenol decreased adenylate cyclase activity minimally.

Additional studies with vinblastine and nocodazole, inhibitors of microtubule assembly, demonstrated an inhibition of vasopressin and cyclic AMP-stimulated water flow but showed no effect on urea transport.

We would conclude that water and urea transport, as examples of hormone-stimulated processes, have different links to cell metabolism, and that in addition to cyclic AMP, a non-nucleotide pathway may be involved in the action of vasopressin.

**Key words: vasopressin, nocodazole, urea transport, rotenone, dinitrophenol, methylene blue**

The peptide hormone vasopressin is the major water-conserving hormone in vertebrates. Upon binding to receptors in the basolateral membrane of its target cell, it activates adenylate cyclase, and, through a series of cyclic AMP-mediated steps, increases the permeability of the contralateral luminal membrane to water [1].

The complexity of the permeability response varies from species to species, and may even vary in adjacent segments of the renal collecting duct. In the cortical segment of the collecting duct, for example, the response is highly selective, since water alone penetrates the cell membrane at a faster rate following vasopressin. There is no change in the permea-

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bility of molecules even as small as urea [2]. In amphibian tissues, notably the urinary bladder, the permeability increase is a broader one, involving water, sodium, urea, and most small hydrophilic and lipophilic molecules. This more complex response may also take place in the medullary segment of the collecting duct, although the experimental evidence remains contradictory [3, 4].

The broader type of membrane response has been of particular interest in our laboratory, since it brings up a number of questions in the area of "transmembrane signaling." Do water and solutes traverse the luminal membrane via a single common pathway, or via independent pathways, each with distinct characteristics? If there are independent pathways, are they activated simultaneously and in the same fashion by cyclic AMP? Is there regulation of the early steps in the adenylate cyclase–cyclic AMP sequence that may direct the mechanism towards water or solute transport? Finally, is hormonal stimulation of water or solute transport mediated exclusively by cyclic AMP, or are other mediating agents involved?

Although many of these questions are unanswered, there is evidence that multiple pathways, rather than a single common pathway, mediate water and solute transport. A variety of agents, including phloretin, permanganate, and chromate, block vasopressin-stimulated urea transport in the toad bladder but have no effect on osmotic water flow [5, 6]. Since these agents are equally effective in blocking urea transport in bladders treated with cyclic AMP rather than vasopressin, they are presumed to act at a distal step in the cyclic nucleotide sequence, probably at the luminal membrane itself. Certain rapidly acting anesthetic agents (including methohexital and methoxyflurane) have an action opposite to that of phloretin and the oxidizing agents: inhibition of water flow with no effect on urea transport [7]. The inhibitory effect of these anesthetic agents can be overcome with exogenous cyclic AMP, suggesting that their action is at a relatively early step in the sequence. This is supported by the finding that methohexital and methoxyflurane almost completely block stimulation of toad bladder adenylate cyclase by vasopressin as well as the hormone-induced rise in cyclic AMP production [8]. In addition to these studies with selective inhibitory agents, we have found that urea and water transport are stimulated in sequence, with urea transport being more responsive than water to low concentrations of vasopressin [9].

There is evidence, then, for separation of cyclic nucleotide-mediated transport systems in the tissue under study, both at the final (membrane) step in the sequence, and possibly at earlier steps as well. In the studies to be presented, the concept of separate pathways is developed further, with the demonstration that the control of hormonally stimulated water and urea transport may be linked in distinctive ways to cell metabolism, and that microtubule assembly appears to mediate water transport but not urea transport.

## MATERIALS AND METHODS

Studies were performed in paired bladder sacs removed from doubly pithed Dominican toads (Bufo marinus, National Reagents, Bridgeport, CT). These were tied to glass bungs and washed three times in phosphate-buffered amphibian Ringer's solution (120 mM $Na^+$, 4.0 mM $K^+$, 0.5 mM $Ca^{2+}$, 116 mM $Cl^-$, 1.0 mM $H_2PO_4^-$, 4.0 mM $HPO_4^{2-}$, pH 7.4) to remove endogenous vasopressin. Control bladder halves were filled with Ringer's diluted 1:10 with distilled water and containing tracer amounts of $^{14}C$ urea (New England Nuclear Co.). Sacs were suspended in beakers containing 35 ml full-strength Ringer's. The paired test bladder halves were treated in identical fashion, except that the test inhibitor

was added to the serosal solution. In the rotenone studies, the Ringer's solution contained 0.1–1% ethanol to maintain the rotenone in solution. The control bladder half was also bathed in Ringer's containing 0.1–1% ethanol. Air was bubbled through serosal baths, and continuous stirring was provided inside and out by rotating bar magnets. Following equilibration, permeability coefficients ($K_{trans}$) of $^{14}C$ urea from lumen to serosa were determined for one 15-min period prior to the addition of vasopressin (baseline period), and for two consecutive 15-min periods following the addition of 86 mU/ml vasopressin (Sigma) to the serosal solution. Osmotic water flow was determined by serially weighing the bladders. The above protocol was modified for experiments involving cyclic AMP or 8-Br-cAMP: after one 15-min baseline period, sacs were placed in fresh serosal medium containing the nucleotide. Two consecutive 15-min periods were then determined and the results pooled for ease of presentation. In all experiments, results in the test bladder half were compared to the control half by the method of pair analysis [10].

In studies of vinblastine and nocodazole, the protocol described by Taylor and coworkers [11] was employed. The Ringer's solution contained 0.9 mM $Ca^{++}$ at pH 7.3, and the mucosal bath was again diluted 1:10. Control and experimental sacs were suspended in 35 ml serosal solutions and air was bubbled serosally for a 4 h incubation period with vinblastine or nocodazole in the serosal medium of the test sacs. $^{14}C$ urea was then added to the mucosal solution, and the standard baseline and post-vasopressin studies were carried out. In the vinblastine studies, the test serosal bath contained 1% ethanol in order to maintain the vinblastine in solution. Control serosal baths contained 1% ethanol as well. For experiments with 12 mM cAMP and 8-Br-cAMP, the mucosal solution volume was 6 ml, and the serosal volume 18 ml. Stirring was employed inside and outside the sacs following the 4 h incubation period.

Short-circuit current was determined in test and control bladder quarters in a divided lucite chamber employing the method of Ussing and Zerahn [12].

Adenylate cyclase activity was determined by a modification of the method of Salomon, Londos and Rodbell [13], in which a particulate fraction of isolated homogenized toad bladder epithelial cells was employed. The incubation was carried out for 15 min in 10 × 75 mm test tubes in a final volume of 50 $\mu$l of 25 mM Tris HCl, pH 7.6, 30 mM KCl, 5 mM $MgCl_2$ 1.4 mM EDTA, 1 mM cAMP (Sigma), 1 mM $\alpha$-$^{32}$P-ATP (2–4 × $10^6$ cpm, New England Nuclear), 0.5 mg/ml bovine serum albumin (Sigma) and an ATP-regenerating system consisting of 1 mg/ml creatinine kinase (155 units/mg) (Sigma), and 20 mM phosphocreatine (Sigma). Control tubes contained enzyme alone (designated I in Table II); tubes designated II contained enzyme plus 100 mU/ml vasopressin (control bladder) or 100mU/ml vasopressin plus inhibitory agent (experimental bladder). Following incubation, the samples were chromatographed to isolate $^{32}$P cyclic AMP.

Intracellular cyclic AMP levels for the rotenone-treated bladders were determined by a modification of the method of Omachi et al [14]. Excised bladders were washed in Ringer's. Each hemibladder was divided into two approximately equal parts, for a total of 4 tissue segments per toad. The segments were washed twice more with Ringer's and then separately incubated in Ringer's containing 10 mM theophylline for 30 min at room temperature (preparatory period). One of the 4 segments then continued to incubate in Ringer's plus theophylline (control bladder, baseline assay); a third received rotenone (test bladder, baseline assay); and a fourth received rotenone plus vasopressin (test bladder, vasopressin assay).

Rotenone was kindly supplied by Dr. Henry Hoberman (Dept of Biochemistry, Albert Einstein College of Medicine); dinitrophenol, Matheson, Coleman and Bell;

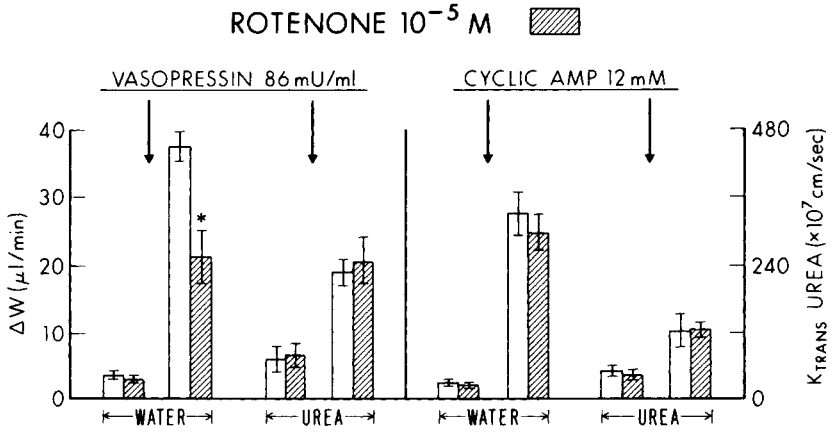


Fig. 1. Effect of rotenone (hatched bars) on water flow and urea transport in bladders stimulated by vasopressin (left panel) and cyclic AMP (right panel). * = significant effect; vertical lines show ± 1 SEM.

methylene blue, J.T. Baker Chemical Co.; vinblastine, Sigma Chemical Co., nocodazole [methyl[5-2(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate, R17934], Aldrich Chemical Co.; cAMP and 8-Br-cAMP, Sigma Chemical Co.

## RESULTS

In a series of experiments in which water and urea transport were determined in paired bladder sacs, one of which was treated with a metabolic inhibitor, the other serving as a control, selective inhibition of water or urea transport was demonstrated.

The effect of $10^{-5}$ M rotenone in the serosal bathing medium is shown in Fig. 1. There was no effect on water flow or urea transport in the absence of vasopressin. Following vasopressin, the usual large increase in water flow took place in the control bladders, while the rotenone-treated bladders showed a significant (47%) reduction in the extent of vasopressin stimulation of water flow. Rotenone had no effect on urea transport. When 12 mM cyclic AMP was substituted for vasopressin (Fig. 1, right-hand panel), there was no inhibitory effect of rotenone on either transport process. Similar results were obtained with amytal, which produced a 21% inhibition of vasopressin-stimulated water flow ($P < 0.01$), but had no effect on cyclic AMP-stimulated water flow, and no effect on either vasopressin- or cyclic AMP-stimulated urea transport.

Figure 2 shows the relationship of rotenone concentration in the serosal bathing medium to water and urea transport. Rotenone was an effective inhibitor of water transport in concentrations as low as $10^{-6}$ M. It was virtually as effective in inhibiting vasopressin-stimulated water flow when placed in the luminal bathing medium: inhibition was 25% at $10^{-6}$ M, and 40% at $10^{-5}$ M, with no effect on urea transfer.

In the toad bladder short-circuit current is an accurate index of active sodium transport from lumen to serosa [12]. Following vasopressin, short-circuit current characteristically rises to more than twice its baseline value. In experiments carried out in a divided lucite chamber in which one bladder quarter acted as a control for the quarter exposed to $10^{-6}$ M rotenone in the serosal medium, rotenone inhibited the post-vasopressin increase in short-circuit current by 56% ($n = 6$, $P < 0.05$).

Dinitrophenol, $10^{-4}$ M in the serosal bathing medium, exhibited virtually the same

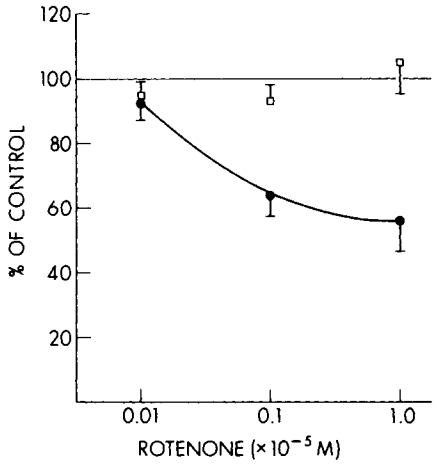


Fig. 2. Dose-response curve for rotenone inhibition of water flow (•). □ = urea transport.

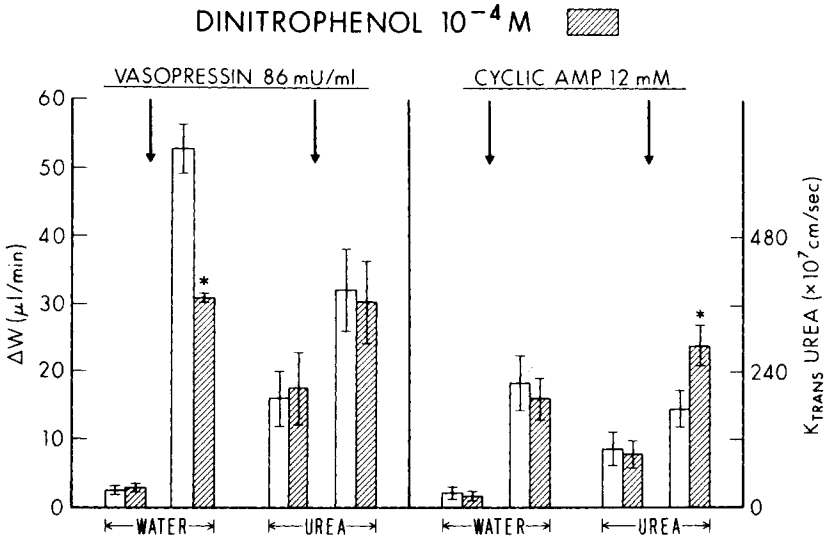


Fig. 3. Effect of dinitrophenol on water and urea transport.

pattern of inhibition as rotenone (Fig. 3). Vasopressin-stimulated water flow was inhibited by almost 50%, with no effect on urea transport. Dinitrophenol had no inhibitory effect on cyclic AMP-stimulated water flow, and actually accelerated cyclic AMP-stimulated urea transport. Dinitrophenol decreased both baseline and vasopressin-stimulated short-circuit current.

Methylene blue, $5 \times 10^{-4}$ M, in contrast to the above agents, selectively inhibited vasopressin-stimulated urea transport, with no effect on osmotic water flow. Its inhibitory effect was not seen in cyclic AMP-stimulated bladders (Fig. 4). The selective inhibitory effect of methylene blue on urea transport was only evident when the bladder was placed in the luminal bathing medium; serosal methylene blue depressed both water flow and urea transport over a concentration of $10^{-6}$ to $10^{-5}$ M.

**TABLE I. Effects of Metabolic Inhibitors on 8-Br-cAMP-Stimulated Water Flow and Urea Transport**

| Agent | Control bladder | | | Experimental | | | Net Δ (Cont-Exp) |
|---|---|---|---|---|---|---|---|
| | I* | II | Δ | I | II | Δ | |
| | A. Water Flow (μl/min) | | | | | | |
| Rotenone (5) | 2.9 | 53.9 | 51 | 2.0 | 45.1 | 43.1 | 7.9 ± 2.3† |
| Dinitrophenol (6) | 3.3 | 48.0 | 44.7 | 3.3 | 49.8 | 46.5 | − 1.8 ± 4.5 |
| Methylene blue (4) | 2.5 | 53.5 | 51.0 | 2.8 | 51.0 | 48.2 | 2.8 ± 2.0 |
| | B. $K_{trans}$ urea ($10^7$ cm/sec) | | | | | | |
| Rotenone (5) | 162 | 387 | 225 | 201 | 415 | 214 | 11 ± 14 |
| Dinitrophenol (6) | 43 | 132 | 89 | 60 | 337 | 277 | − 188 ± 42† |
| Methylene blue (4) | 111 | 440 | 329 | 131 | 466 | 335 | − 6 ± 49 |

*Period I, baseline; period II, following 1.5 mM 8-Br-cAMP.
†Significant difference ($p < 0.05$ or better).

In the experiments so far described, exogenous cyclic AMP stimulated water and urea transport less than did vasopressin. Therefore, an additional series of studies was carried out using 1.5 mM 8-Br-cAMP, a potent analogue of cAMP. The results are shown in Table I. Water flow exceeded, and urea transport was comparable to that produced by vasopressin. There was a small (15%) inhibition of the increment in water flow by rotenone, far less than the 47% seen in the vasopressin series. Dinitrophenol did not inhibit water flow, and as with cAMP, stimulated urea transport. Methylene blue had no effect on water or urea. Thus, the findings with 8-Br-cAMP were comparable to cAMP.

The three agents studied, rotenone, dinitrophenol, and methylene blue, all inhibited a particular vasopressin-stimulated transport process. The inhibition was not apparent when cyclic AMP was substituted for vasopressin, and there was only a small inhibition of water flow by rotenone when 8-Br-cAMP was employed. The findings suggest that, to the extent that the cyclic nucleotide system is involved at all in the action of these inhibitors, their effect is largely at a step prior to the generation of cyclic AMP. A precedent for this type of action is found in earlier studies of the rapidly acting anesthetics methohexital and methoxyflurane, which selectively inhibited vasopressin-stimulated, but not cyclic AMP-stimulated water flow [7]. Here, significant reductions in adenylate cyclase activity and intracellular cyclic AMP levels could be demonstrated [8]. However, as shown in the upper half of Table II, the reduction in adenylate cyclase activity by rotenone was only 12% and of borderline significance, whereas dinitrophenol reduced adenylate cyclase by only 7%. There was no decrease in cyclic AMP levels in bladders treated with rotenone (lower half of Table II). Preliminary studies show no decrease in cyclic AMP levels in methylene blue-treated bladders [Hays, Franki and Ross, unpublished observations].

In the final group of experiments to be reported, we determined the extent to which microtubule assembly was involved in the stimulation of urea transport. Earlier reports by Taylor and co-workers [11] indicated that vasopressin or cyclic AMP-stimulated water flow was inhibited by colchicine and vinblastine. Their conclusion that cytoplasmic microtubules were indeed associated with the action of vasopressin on water flow was supported by a number of kinetic and morphologic studies [15].

Figure 5 shows the effect of $10^{-6}$ M vinblastine on water and urea transport in

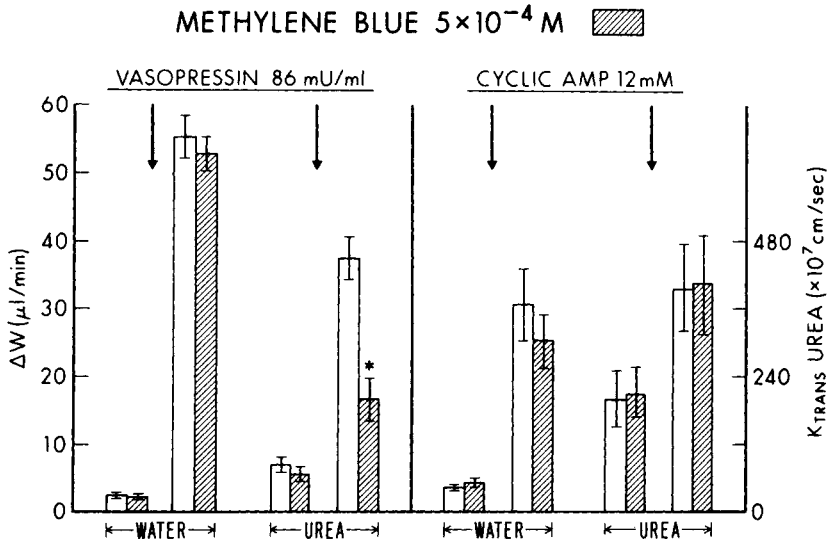


Fig. 4. Inhibition of vasopressin-stimulated urea transport by methylene blue.

bladders receiving 20 mU/ml vasopressin (a near-saturating concentration employed in earlier studies [11]), or 12 mM cyclic AMP. Vinblastine inhibited water flow following both vasopressin and cyclic AMP, but had no effect on urea transport. $10^{-6}$ M nocodazole, a synthetic inhibitor of tubulin polymerization [16], also inhibited vasopressin-stimulated water flow (32% inhibition, $P < 0.01$) with no effect on urea transport.

## DISCUSSION

Rotenone, dinitrophenol, and methylene blue, agents which interfere with oxidative metabolism, show distinctive effects on vasopressin-stimulated water and urea transport. Rotenone, a toxic plant substance which blocks mitochondrial electron transport between NADH and ubiquinone, blocked hormone-stimulated water flow with no effect on urea transport. Amytal, which has an action identical to rotenone, had the same selective effect on water and urea transport. Dinitrophenol, an uncoupler of oxidative phosphorylation,

**TABLE II. Effects of Metabolic Inhibitors on Vasopressin-Stimulated Adenylate Cyclase and Cyclic AMP**

| Agent | Control bladder | | | Experimental bladder | | | Net Δ (Cont-Exp) | P |
|---|---|---|---|---|---|---|---|---|
| | I* | II | Δ | I | II | Δ | | |
| | A. Adenylate Cyclase (pM cAMP/mg prot/15 min) | | | | | | | |
| Rotenone (9)† | 1306 | 2394 | 1088 | 1238 | 2187 | 949 | 139 ± 75 | < 0.1 |
| Dinitrophenol (4) | 1014 | 3080 | 2067 | 999 | 2911 | 1912 | 155 ± 38 | < 0.05 |
| | B. Cyclic AMP (pM/mg prot) | | | | | | | |
| Rotenone (6) | 10.3 | 65.3 | 55.0 | 13.5 | 75.2 | 61.7 | − 6.7 ± 24.2 | NS |

*Assay I, baseline; assay II, following vasopressin (control baldder), or vasopressin plus inhibitor (experimental bladder).

†Numbers in parentheses refer to number of toads.

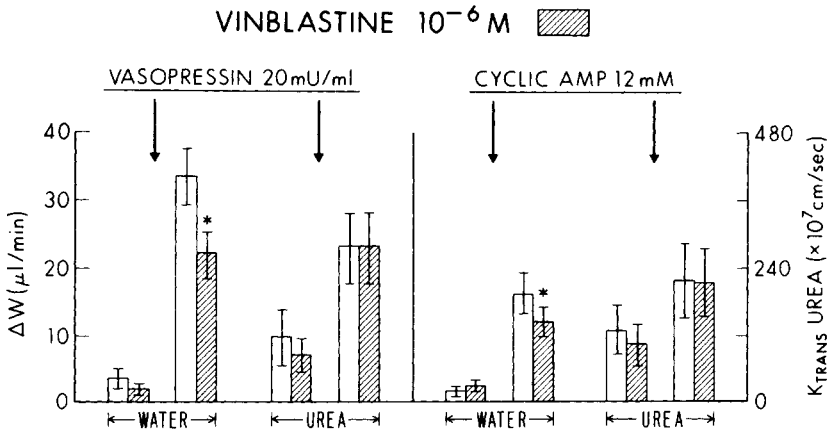


Fig. 5. Inhibition of vasopressin and cyclic AMP- stimulated water flow by vinblastine.

selectively blocked water transport in a pattern comparable to rotenone and amytal. Rotenone and dinitrophenol were selective inhibitors whether they were placed in the serosal or luminal bathing medium; amytal was examined in the serosal medium only.

The significant decline in vasopressin-stimulated active sodium transport produced by rotenone, and in both baseline and vasopressin-stimulated sodium transport produced by dinitrophenol, is consistent with interference in oxidative metabolism by both of these agents, since sodium transport requires a significant fraction of the energy production of this tissue [17]. It is possible, of course, that rotenone, amytal,* and dinitrophenol exert effects in the toad bladder other than their well-characterized ones on oxidative metabolism; these cannot be evaluated until additional studies are carried out.

The inhibitory action of both rotenone and dinitrophenol on water flow was not evident when cyclic AMP was substituted for vasopressin; a small inhibition of water flow by rotenone was seen when 8-Br-cAMP was used. These findings suggest that both rotenone and dinitrophenol inhibit water flow largely prior to the generation of cyclic AMP, to the extent that their action involves the cyclic nucleotide system at all. A second site of action for rotenone, beyond the generation of cAMP, is possible. Of interest is the actual acceleration of cyclic AMP-stimulated urea transport by dinitrophenol. This remains unexplained, but again emphasizes the important differences that exist in the control of both proximal and distal components of the water and urea pathways.

Our findings with methylene blue, an agent which interrupts mitochondrial electron transport by accepting electrons from NADH, complement those with rotenone and dinitrophenol. Methylene blue inhibits vasopressin-stimulated, but not cyclic AMP or 8-Br-cAMP-stimulated urea transport, and has no effect on water flow. Thus, it is presumed to act at an early step in the hormone-activated urea transport pathway. Unlike rotenone and dinitrophenol, methylene blue is a specific inhibitor of urea transport only when it is placed in the luminal bathing medium; it inhibits both water and urea transport when placed in the serosal medium. This finding suggests that passage of the methylene blue across the luminal border of the cell and across the cytoplasm to the basolateral surface, where adenylate cyclase is presumably located, is important in terms of its selective inhibition of urea transport. Whether methylene blue is altered by the cell, or its cytoplasmic

*Amytal is also a barbiturate, and it is possible that it shares the mechanism of action of other barbiturates already referred to (8), ie, significant inhibition of adenylate cyclase. This possibility was not tested in this paper.

concentration is altered to permit a selective action on urea transport is unclear. Alternatively, the intracellular site "prior to the generation of cyclic AMP" may, in the case of urea, be a more luminal site than that for water. We also recognize the possibility that methylene blue, like rotenone and dinitrophenol, may inhibit urea transport by a mechanism other than its interference with electron transport. It may act as a less specific oxidizing agent; selective interference with urea transport has been found in studies with chromate and permanganate, for example [6].

What are the intracellular sites at which these metabolic inhibitors exert their action? This question has not been explored to any extent in the experiments reported in this paper. Adenylate cyclase activity was decreased to a very small extent by rotenone and dinitrophenol (Table II). The decrease may be significant, however, since there is evidence that the "cyclic AMP pool" involved in the permeability response of the toad bladder may be a very small fraction of total intracellular cyclic AMP [18, 19].

If the adenylate cyclase response to vasopressin is indeed decreased by metabolic inhibitors, what is the link between oxidative metabolism and adenylate cyclase activity? It is unlikely that ATP levels are depressed to the point that ATP is rate-limiting as a substrate for conversion to cyclic AMP; the Kṁ for toad bladder adenylate cyclase is approximately $10^{-4}$M [20], and suppression of ATP synthesis would have to be virtually complete for this to be the mechanism. However, there is growing recognition that adenylate cyclase is an extraordinarily complex enzyme and that a large number of cytoplasmic factors are involved in its activation [21]. ATP may play an important role as an allosteric factor [22] in maintaining the level of other factors such as GTP [23] or in other ways that are not yet recognized.

The inhibited step in the cyclic nucleotide sequence may not be adenylate cyclase at all, but some other regulatory step of importance in the generation of cyclic AMP. Prostaglandins, for example, are potent inhibitors of vasopressin-stimulated water flow [24] and appear to be significantly less potent in depressing urea transport [Carvounis, Franki and Hays, unpublished]. Acceleration of prostaglandin synthesis, therefore, could selectively depress water flow.

It is also possible that the pathways altered by metabolic inhibitors do not involve the adenylate cyclase–cyclic AMP sequence, but are important regulatory pathways in parallel with the cyclic nucleotide system. In support of this possibility is the failure to observe any decrease in intracellular cyclic AMP levels in rotenone-treated bladders despite a significant decrease in water flow. While it could be argued that this simply reflects the small size of the cyclic AMP pool involved in water flow, it is also consistent with a second regulatory pathway. It is of interest in this connection that the inhibitors under study depressed water or urea transport in a selective fashion by approximately 50%; when high concentrations of the agents were employed, water and urea transport were jointly inhibited. It is possible that water and urea transport have both common and separate activating systems, one involving cyclic nucleotides and the other a non-nucleotide-mediated system.

Turning to the studies of the effect of vinblastine and nocodazole, inhibitors of microtubule aggregation, there was a selective inhibition of both vasopressin- and cyclic AMP-stimulated water flow, but no effect on urea transport. This finding confirms and amplifies the recent report of Taylor and co-workers [25]. The findings suggest that microtubules are not involved in the hormonal activation of urea transport, and point to another major difference in the pathways under study. We have shown in earlier studies that the aggregation of luminal membrane-associated particles that follows hormonal stimulation

of the toad bladder is associated with water flow and does not appear to be related to urea or sodium transport [26]. It is not clear whether these particle aggregates are the result of lateral migration of membrane proteins in the plane of the membrane, or of exocytosis, where a submembrane structure becomes incorporated into the membrane. Microtubules might be involved in the aggregation phenomenon, although their exact role has not been established in this or other systems. Urea entry into the cell would appear to be via some other transmembrane structure.

## ACKNOWLEDGMENTS

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