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Selective Inhibition of Urea Transport by Oxidizing Agents

Evidence for a Site of Inhibition Beyond the Generation of Cyclic AMP

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Summary. Urea and water transport across the toad bladder epithelial cell appears to take place through independent vasopressin-stimulated pathways. Agents such as chromate, for example, when added to the luminal bathing medium, inhibit urea transport without inhibiting osmotic water flow, providing evidence for such independent pathways. In the present study, selective inhibition of urea transport is shown for permanganate and methylene blue, which, like chromate, are oxidizing agents. Permanganate inhibits urea transport irreversibly, while methylene blue acts reversibly. Not all oxidizing agents are inhibitory; perchlorate, peroxide and ferricyanide have no effect on urea transport or water flow. Permanganate and chromate both act at a point beyond the generation of cyclic AMP, since they continue to inhibit urea transport in bladders treated with exogenous cyclic AMP, 8-bromoadenosine 3′,5′- cyclic monophosphate, and a combination of cyclic AMP and theophylline. These findings suggest that selective inhibition of urea transport can be brought about by oxidation of one or more components in its transport pathway, and that, in the case of chromate and permanganate, these components may be in the luminal membrane itself.

Vasopressin increases the permeability of the toad bladder to water, and to urea and many other small solutes. There is strong evidence that this action of the hormone is mediated by cyclic AMP (Orloff & Handler, 1962) and is brought about by an alteration in the luminal membrane of the epithelial cell (Maffly, Hays, Lamdin & Leaf, 1960; Hays & Leaf, 1962; Civan & DiBona, 1974). Recent studies indicate that separate vasopressin-stimulated pathways exist for osmotic water flow and for the movement of certain small solutes. Phloretin, for example, inhibited the movement of amides, thiourea and formaldehyde across the bladder, but had no effect on osmotic water flow, short-circuit cur-

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rent, or the movement of ethanol or ethylene glycol (Levine, Franki & Hays, 1973). The inhibitory action of phloretin was reversible; removal of phloretin from the luminal bathing medium restored urea movement to its normal level. Two other agents, tannic acid and chromate, were also found to inhibit urea movement across the bladder, with no effect on water flow (Shuchter, Franki & Hays, 1973). In contrast to phloretin, the effect of chromate was irreversible. In view of its potent oxidizing ability, it appeared possible that chromate inhibited urea transport by irreversibly oxidizing some component in the urea transport pathway. Whether this component was on the luminal membrane or at an intracellular site was not determined.

In the present study, we have surveyed a number of oxidizing agents, and have found that some, but not all, selectively inhibit urea transport. Two of these, chromate and permanganate, whose site of action was tested, exerted their inhibitory effects at a point beyond the generation of cyclic AMP, a finding consistent with an action at the luminal membrane. Finally, within the group of agents selectively inhibiting urea transport, there were differences in their patterns of action, suggesting effects at different sites in the transport pathway.

Materials and Methods

Paired bladder sacs removed from Dominican toads (*Bufo marinus*, National Reagents, Bridgeport, Conn.) were tied to glass bungs and washed in phosphate-buffered amphibian Ringer's solution consisting of (mM): 120 Na^+, 4.0 K^+, 0.5 Ca^{++}, 116 Cl^-, 1.0 $H_2PO_4^-$, 4 $HPO_4^=$, at pH 7.4, 230 mOsm/Kg H_2O for 20 min in order to remove any endogenous vasopressin. The control bladder half was filled with 6 ml of Ringer's solution diluted 1:10 with distilled water. In all experiments, except those involving chromate, the pH of the luminal bathing medium was maintained at 7.4 with the phosphate buffer; luminal pH was monitored with each new agent used and remained unchanged throughout the experiment. In the chromate experiment, pH was maintained at pH 5.0 with 2 mM potassium phthalate. The luminal solution contained tracer amounts of ^{14}C urea (New England Nuclear Corp., Boston, Mass.). The sacs were suspended in beakers containing 35 ml full-strength Ringer's. The paired test bladder half was treated in the identical fashion, except that the mucosal solution contained the appropriate concentration of test agent. Air was bubbled through the serosal bath and stirring was provided in the outside bathing medium by rotating bar magnets. Following equilibration, the permeability coefficients (K_{trans}) of ^{14}C urea were determined from lumen to serosa for one 15-min period prior to vasopressin addition and two 15-min periods following 86 mU/ml vasopressin. Isotope counting was done in a Tri-carb liquid scintillation spectrometer (Packard Instrument Co., LaGrange, Ill.). Osmotic water flow was determined gravimetrically (Bentley, 1958). Short-circuit current was determined in lucite chambers with a central dividing partition (Sharp & Leaf, 1964). In all experiments, results obtained in the test bladder were compared to the control bladder by the method of paired analysis (Snedecor & Cochran, 1967). It should be noted that although some of the results are presented as percent changes

from control (*see* Table 1, for example), statistical analyses were uniformly done on the absolute difference between control and test water flow and K_{trans} urea (in cm·sec^{-1}) (Maffly *et al.*, 1960).

There were variations in the preceding protocol depending upon the test agent used. In experiments involving the use of cyclic AMP, 8-bromoadenosine 3′,5′-cyclic monophosphate (hereafter abbreviated as 8-Br-cAMP) (Sigma Chemical Co., St. Louis, Mo.) or a combination of cyclic AMP and theophylline, the volume inside the sacs was 5 ml and the volume of the serosal medium was 15 ml. Only air bubbling of the serosal medium was provided, to prevent the rotating magnet from hitting the sac. After one 15-min control period, sacs were placed in fresh serosal medium containing cyclic AMP, 8-Br-cAMP, or a combination of cyclic AMP and theophylline. Two consecutive 15-min periods were then determined. In the theophylline experiments, after one 15-min control period, the sacs were placed in 35 ml of fresh serosal medium containing theophylline, and two consecutive 15-min periods were determined. In presenting the results, the consecutive 15-min periods were pooled into a single 30-min period.

Results

Effect of Oxidizing Agents on Osmotic Water Flow and Urea Permeability

The effect of a series of oxidizing agents on vasopressin-stimulated osmotic water flow and urea permeability is shown in Table 1. Of the agents listed in the Table, chromate, permanganate and methylene blue caused a significant inhibition of urea movement with no effect on osmot-

Table 1. Effect of oxidizing agents on water flow and urea K_{trans}

Oxidizing agents	Concentration (M)	% change, K_{trans} urea; p value	% change, water flow; p value
Chromate	5×10^{-4} (10)[a]	-63 ± 6[b] (<0.001)	-5 ± 3 (NS)
Permanganate	2×10^{-5} (6)	-68 ± 11 (<0.01)	-2 ± 2 (NS)
Methylene blue	5×10^{-4} (6)	-38 ± 9 (<0.05)	1 ± 5 (NS)
Perchlorate	5×10^{-4} (5)	-8 ± 8 (NS)	-3 ± 2 (NS)
	10^{-3} (6)	-1 ± 8 (NS)	-1 ± 2 (NS)
	5×10^{-3} (5)	-13 ± 8 (NS)	-3 ± 2 (NS)
Peroxide	10^{-4} (2)	6 ± 4 (NS)	7 ± 8 (NS)
	5×10^{-4} (2)	-3 ± 7 (NS)	-6 ± 2 (NS)
	10^{-3} (4)	-4 ± 3 (NS)	-6 ± 4 (NS)
Ferricyanide	10^{-4} (1)	14	-14
	10^{-3} (3)	-1 ± 6 (NS)	5 ± 6 (NS)
	5×10^{-3} (1)	19	2

[a] Numbers in parentheses indicate number of experiments.
[b] ±1 SEM.

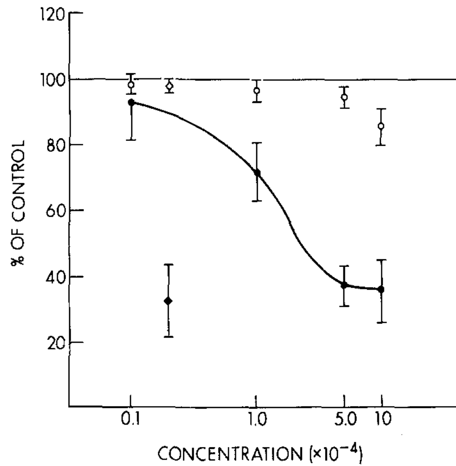

Fig. 1. Dose-response curve for effect of chromate on K_{trans} urea (●) and osmotic water flow (○). A single point is shown for the effect of 2×10^{-5} M permanganate on K_{trans} urea (◆) and osmotic water flow (◇). Data are expressed as per cent of paired control K_{trans} urea and water flow

Table 2. Effect of chromate and permanganate on vasopressin-induced water flow and K_{trans} urea

	Control bladder		Test bladder		Δ (Control-Test); *p* value	
	I[a]	II	I	II	I	II
5×10^{-4} M chromate (10)[b]						
Water[c]	3.3±0.2[e]	47.9±1.8	3.4±0.2	45.3±1.4	−0.1±0.2 (NS)	2.6±1.4 (NS)
Urea ^{14}C[d]	43±13	298±27	61±17	114±23	−18±12 (NS)	184±20 (<0.001)
2×10^{-5} M permanganate (6)						
Water	3.6±0.4	45.7±2.1	3.1±0.2	45.1±2.3	0.5±0.4 (NS)	0.6±0.8 (NS)
Urea ^{14}C	124±25	451±49	117±27	136±41	7±17 (NS)	315±67 (<0.01)

[a] Period I, 15 min; Period II, 30 min, in this and following tables; bladders were exposed to vasopressin, or (in following tables) cyclic AMP and/or theophylline after Period I.
[b] Numbers in parentheses indicate number of experiments.
[c] In μliter per min per sec in this and following tables.
[d] In cm per sec × 10^7 in this and following tables.
[e] ±1 SEM.

ic water flow. Perchlorate, peroxide and ferricyanide had no significant effects on either osmotic water flow or urea movement.

The dose-response relationship for two of the agents, chromate and permanganate, is shown in Fig. 1. A full dose-response curve is shown for chromate, and a single point for permanganate. Permanganate was

clearly the more potent agent of the two, inhibiting K_{trans} urea by 68%, at a concentration of 2×10^{-5} M. A comparable inhibition by chromate required a chromate concentration between 1 and 5×10^{-4} M. Water movement was unimpaired at all experimental points for both ions, including 10^{-3} M chromate. The actual data for vasopressin-stimulated K_{trans} urea and osmotic water flow in the presence of 5×10^{-4} M chromate and 2×10^{-5} M permanganate are shown in Table 2.

Effects of Chromate and Permanganate on Cyclic Nucleotide-Treated Bladders

In the series of experiments to follow, the effects of chromate and permanganate, the two most potent inhibitory agents, were determined on bladders treated with cyclic nucleotides and a combination of cyclic AMP and theophylline. The effect of 5×10^{-4} M chromate is shown in Table 3. Following 24 mM cyclic AMP, both water flow and K_{trans} urea in control bladders reached levels seen following vasopressin, and there was a significant decrease in K_{trans} urea (47%, $p < 0.02$) in the chromate-treated bladders. Water flow was not significantly depressed. In view of the fact that very large concentrations of cyclic AMP were required to give maximal control water flow and urea movement, the effects of 8-Br-cAMP, a potent analogue of cyclic AMP, and a combination of 6 mM cyclic AMP and 3 mM theophylline on paired bladders were determined. The effect of chromate and permanganate on 8-Br-cAMP-treated bladders is shown in Table 4. There was a marked inhibition of K_{trans} urea by chromate (76% inhibition) and by permanganate (94% inhibition), with no significant inhibition of water flow in either case.

The effects of chromate and permanganate on bladders treated with a combination of 6 mM cyclic AMP and 3 mM theophylline were then determined. These concentrations of cyclic AMP and theophylline, when

Table 3. Effect of 5×10^{-4} chromate on cyclic AMP-induced water flow and K_{trans} urea

	Control bladder		Test bladder		Δ (Control-Test); p value	
	I	II	I	II	I	II
24 mM cyclic AMP (8)						
Water	2.5±0.2	43.3±2.4	3.1±0.3	39.9±2.1	−0.6±0.3 (NS)	3.4±2.5 (NS)
Urea ^{14}C	94±12	314±55	118±23	165±29	−24±13 (NS)	149±44 (<0.02)

Table 4. Effect of chromate and permanganate on 8-Br-cAMP[a]-induced water flow and K_{trans} urea

	Control bladder		Test bladder		Δ (Control-Test); *p* value	
	I	II	I	II	I	II
5×10^{-4} M chromate (4)						
Water	2.25±0.2	48.5±1.8	2.27±0.4	44.7±2.8	−0.02±0.5 (NS)	3.8±2.0 (NS)
Urea ^{14}C	34±13	374±49	23±10	89±39	11±5 (NS)	285±28 (<0.01)
2×10^{-5} M permanganate (4)						
Water	2.6±0.3	50.0±3.5	2.5±0.2	46.7±2.9	0.1±0.3 (NS)	3.3±2.4 (NS)
Urea ^{14}C	7±2	401±64	9±3	24±9	−2±1 (NS)	377±63 (<0.01)

[a] 1.5 mM 8-Br-cAMP used in all experiments.

Table 5. Effect of chromate and permanganate on combined cyclic AMP+theophylline[a] induced water flow and K_{trans} urea

	Control bladder		Test bladder		Δ (Control-Test); *p* value	
	I	II	I	II	I	II
5×10^{-4} M chromate (4)						
Water	3.0±0.2	37.1±5.9	3.3±0.5	35.3±2.7	−0.3±0.4 (NS)	1.8±4.2 (NS)
Urea ^{14}C	111±42	367±52	169±66	269±60	−58±24 (NS)	98±22 (<0.05)
2×10^{-5} M permanganate (6)						
Water	3.9±0.5	68.1±3.3	4.1±0.3	69.5±2.8	−0.2±0.5 (NS)	−1.4±3.3 (NS)
Urea ^{14}C	60±43	381±88	50±35	84±24	10±8 (NS)	297±68 (<0.01)

[a] 6 mM cyclic AMP+3 mM theophylline-used in all experiments.

Table 6. Effect of 5×10^{-4} M chromate on theophylline-induced water flow and K_{trans} urea

	Control bladder		Test bladder		Δ (Control-Test); *p* value	
	I	II	I	II	I	II
10 mM theophylline (9)						
Water	4.1±0.3	18.0±2.2	3.3±0.3	20.1±2.6	0.8±0.4 (NS)	−2.1±2.2 (NS)
Urea ^{14}C	94±23	270±48	125±25	176±30	−31±20 (NS)	94±31 (<0.02)
25 mM theophylline (4)						
Water	2.8±0.4	24.6±3.6	3.5±0.3	23.5±2.4	−0.7±0.3 (NS)	1.1±1.2 (NS)
Urea ^{14}C	17±5	336±30	30±7	168±14	−13±5 (NS)	168±20 (<0.01)

used alone, gave values for water flow and K_{trans} urea in control bladders which were considerably below those obtained with vasopressin (11 to 54% of vasopressin values: Levine & Worthington, *unpublished data*). When these agents were combined, maximal values for water flow and K_{trans} urea were obtained (Table 5). Again, inhibition of K_{trans} urea, with no effect on water flow, was evident with both chromate and permanganate.

Effect of Chromate on Theophylline-Treated Bladders

The effect of 5×10^{-4} M chromate on theophylline-induced water flow and K_{trans} urea is shown in Table 6. Chromate had no effect on osmotic water flow in bladders exposed to 10 or 25 mM theophylline, but significantly inhibited K_{trans} urea.

Patterns of Action of Chromate, Permanganate and Methylene Blue

Previous studies (Shuchter *et al.*, 1973) showed that the inhibition of vasopressin-stimulated urea movement by chromate was irreversible. Permanganate, like chromate, irreversibly inhibited K_{trans} urea (upper half of Table 7). In these experiments, permanganate was placed in the luminal medium for 15 min, then removed, with three subsequent washouts of the luminal medium with Ringer's solution. 1/10 Ringer's solution was then placed in both sacs, and the usual pre- and post

Table 7. Reversibility of inhibition of K_{trans} urea by permanganate and methylene blue

	Control		Test		Δ (Control-Test); p value	
	I	II	I	II	I	II
2×10^{-5} M permanganate (4)[a]						
Water	3.0±0.2	69.1±3.3	3.1±0.1	71.2±2.3	−0.1±0.3 (NS)	−2.1±4.1 (NS)
Urea ^{14}C	166±58	469±84	109±29	252±53	57±36 (NS)	217±66 (<0.05)
5×10^{-4} M methylene blue (5)[b]						
Water	2.3±0.5	62.4±5.5	3.0±0.7	58.3±6.4	−0.7±0.9 (NS)	4.1±3.4 (NS)
Urea ^{14}C	33±10	291±33	84±35	268±60	−51±25 (NS)	23±38 (NS)

The periods shown are those following the removal of permanganate or methylene blue from the luminal medium of the test bladders. Vasopressin added after period I.

[a] Bladders exposed to permanganate for 15 min prior to washout.

[b] Bladders exposed to methylene blue for 60 min prior to washout.

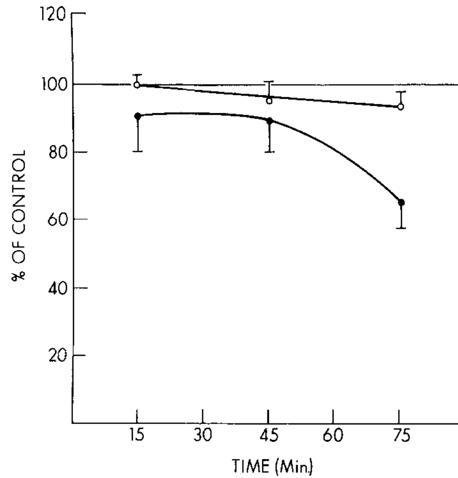

Fig. 2. Effect of 2×10^{-5} M permanganate on K_{trans} urea (●) and osmotic water flow (○) in bladders previously exposed to vasopressin. At time zero, vasopressin added. 15 min later (first points in Figure), K_{trans} urea and water flow determined. Following this, permanganate added to test bladder, and two subsequent half-hour periods run. Results expressed as % of control bladder

vasopressin periods determined. The test bladder, previously exposed to permanganate, showed a large depression of K_{trans} urea, with no inhibition of water flow. Methylene blue, on the other hand, an oxidizing agent which effectively inhibited K_{trans} urea (Table 1), proved to act reversibly. Following removal of methylene blue from the luminal bathing medium, K_{trans} urea rose to levels equal to that of the control bladder (lower half of Table 7).

A major difference in the actions of chromate and permanganate centered around the ability of the two agents to block urea transport if added to bladders already stimulated by vasopressin. Chromate had no inhibitory effect if added after vasopressin (Shuchter *et al.*, 1973). Permanganate, however, inhibited urea movement even when added after vasopressin, or after cyclic AMP plus theophylline. This is shown in Figs. 2 and 3. Here, both control and test bladders were treated with vasopressin (Fig. 2): 15 min later, the test bladder was treated with 2×10^{-5} M permanganate. K_{trans} urea was slightly, but not significantly depressed 30 min after the addition of permanganate; 60 min after permanganate it was significantly depressed (41%, $p < 0.02$). Osmotic water flow was not significantly depressed at 30 or 60 min. A similar pattern was seen in bladders initially stimulated with combined cyclic AMP and theophylline (Fig. 3): here, inhibition of K_{trans} urea was seen both

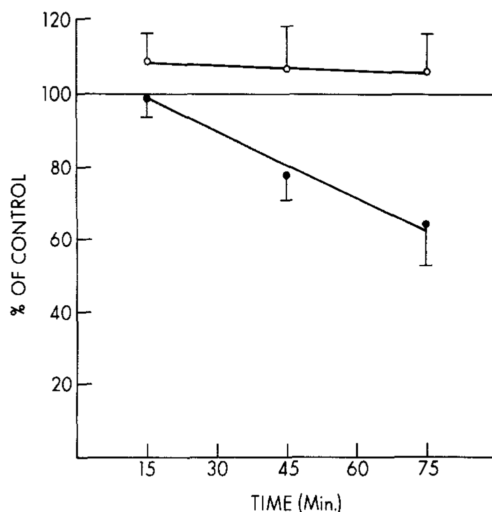

Fig. 3. Effect of 2×10^{-5} M permanganate on K_{trans} urea in bladders previously exposed to 6 mM cyclic AMP and 3 mM theophylline. Protocol the same as Fig. 2

30 and 60 min after the addition of permanganate. Again, there was no inhibition of osmotic water flow.

Effect of Reducing Agents on K_{trans} Urea Following Treatment with Permanganate

To determine whether reducing agents could reverse the inhibitory effects of permanganate on urea transport, both test and control bladders were exposed to 10^{-4} M permanganate, and, in the presence of permanganate, a control period (period I) and a vasopressin period (period II) were run (Table 8, periods I and II). Permanganate was then removed

Table 8. Effect of reducing agents on permanganate-inhibited K_{trans} urea

	Control			Test		
	I[a]	II[b]	III[c]	I	II	III
DTT (2 mM)	64	32	54	48	30	52
Ascorbic Acid (1 mM)	68	34	50	63	32	34
Hydrazine sulfate (0.5 mM)	266	89	74	272	88	50

[a] Period I, 15 min control. Permanganate present.

[b] Period II, 30 min, in the presence of vasopressin (mean of two 15-min periods). Permanganate present.

[c] Period III, 120 min (mean of four 30-min periods). This period follows washout of permanganate and re-exposure to vasopressin (control bladder), and washout of permanganate, introduction of reducing agent into luminal bathing medium and re-exposure to vasopressin (test bladder). All data are means of two experiments.

from the luminal medium of both bladders, and the test bladder was re-exposed to vasopressin and a reducing agent (dithiothreitol, ascorbic acid or hydrazine sulfate) in the luminal medium for a period of 2 hr (period III). The control bladder received vasopressin alone. The permanganate-induced inhibition of urea transport was equally apparent in both test and control bladders, showing no reversal by the reducing agents.

Discussion

The first question taken up in this paper is whether oxidizing agents other than chromate selectively inhibit vasopressin-stimulated urea transport, and whether their action is indeed related to the process of oxidation. Of the six agents listed in Table I, three (chromate, permanganate and methylene blue) inhibited urea transport without reducing water flow. To this group can be added an additional agent, periodate, which has recently been shown to inhibit urea transport selectively (Rubin, 1975). Three of the agents tested (perchlorate, peroxide and ferricyanide) had no effect on urea or water movement, even at 1 to 5×10^{-3} M concentrations.

There is abundant evidence that chromate and/or permanganate can oxidize protein and lipid groups. Cleavage of protein molecules via oxidation of disulfide bridges (Sanger, 1949), or histidine, tryptophan or tyrosine (Witkop, 1961; Hake, 1965; Joly, 1965; Hopwood, 1969), has been reported. The photodynamic oxidation of amino acids by methylene blue has been reported by Knowles and Gurani (1972). Permanganate and periodate, either alone or in combination, also act on lipids. Permanganate-periodate oxidation has been used for the analysis of double bond position in fatty acids (Bloomfield & Bloch, 1960; Scheuerbrandt & Bloch, 1962), as well as for the analysis of saturated lecithins (Shimojo, Abe & Ohta, 1974). Lemieux and von Rudloff (1955) have used a mildly alkaline solution of periodate and permanganate to oxidize olefins to a mixture of carboxylic acids. It is entirely possible, therefore, that membrane proteins or lipids are oxidized by these agents.

The irreversibility of the action of chromate and permanganate is also consistent with oxidation. Not all of the oxidizing agents tested inhibited urea transport, which may simply illustrate the specificity of the oxidative reaction. Exposure of permanganate-treated bladders to three reducing agents, dithiothreitol (DTT), ascorbic acid and hydrazine sulfate, for a period of 2 hr, failed to restore K_{trans} urea to normal

levels (Table 8). The reducing agents themselves had no inhibitory effect on K_{trans} urea, as determined in a separate group of experiments. The failure of the reducing agents to restore the high permeability of the bladder to urea may be the consequence of the irreversibility of the initial oxidation.

While oxidation may be the mechanism of action of chromate, permanganate and methylene blue, other mechanisms have not been ruled out. The formation of stable addition complexes of oxidizing agents with the membrane, for example, would explain our findings equally well. A comparable dilemma was encountered by Ohlsson and Wilson (1974) in their study of alkaline phosphatase inhibition by periodate and permanganate. They demonstrated the inhibition of alkaline phosphatase by periodate and permanganate but not by chromate or perchlorate, at least at low concentration. They were unable to reduce the "oxidized enzyme" with ascorbate, a mild reductant, but were able to restore activity with Mn^{2+}. On the basis of this, they concluded that oxidation might be involved in the inhibition of alkaline phosphatase by permanganate, but felt that the results were also consistent with the formation of a stable addition complex.

Turning to the site of action of chromate and permanganate, our findings indicate that they act beyond the generation of cyclic AMP. Selective depression of K_{trans} urea was demonstrated with cyclic AMP and theophylline, and 8-Br-cAMP. The latter agent, first synthesized by Ikehara and Uesugi (1969), is a potent analogue of cyclic AMP, whose ability to stimulate bovine brain kinase is comparable to that of cyclic AMP (Muneyama, Bauer, Shuman, Robins & Simon, 1971). In addition, it is resistant to phosphodiesterase (Muneyama *et al.,* 1971), and reduces the breakdown of endogenous cyclic AMP by acting as a competitive inhibitor of phosphodiesterase (Harris, Chasin, Phillips, Goldenberg, Samaniego & Hess, 1973).

Does the observation that chromate and permanganate act beyond the generation of cyclic AMP mean that the effect is on the luminal membrane? It is possible that one or more intermediates exist in the cytoplasmic phase between cyclic AMP and the membrane. However, recent studies by Schwartz, Shlatz, Kinne-Saffran and Kinne (1974), have shown that a cyclic AMP-activated kinase is present on the luminal membrane of bovine renal collecting duct cells, suggesting that in this tissue, at least, the step beyond cyclic AMP is in the membrane itself.

Finally, it should be noted that the pattern of action of permanganate differs from that of chromate in at least one important aspect. Previous

studies (Shuchter *et al.*, 1973) have shown that chromate does not inhibit urea permeability if it is added to bladders pretreated with vasopressin. This implies that urea transport pathways already stimulated by vasopressin are resistant to chromate action. It is possible that the component of the pathway which is oxidized by chromate in the absence of vasopressin is not available to chromate following hormonal alteration of the pathway. Permanganate, on the other hand, appears to act on the pathway regardless of its configuration, since it will inhibit urea movement whether added before or after vasopressin, or after a combination of cyclic AMP and theophylline. The extent of the depression was somewhat less than in bladders to which permanganate was added prior to vasopressin, suggesting that permanganate could not modify the urea transport pathway as effectively if added after vasopressin. Methylene blue, as opposed to both chromate and permanganate, is a reversible inhibitor of urea transport. It is not yet established whether methylene blue acts prior to or following the generation of cyclic AMP. These findings suggest that even within the category of oxidizing agents acting on the transport pathway, there are differences in the exact site or mode of action.

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