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Control of Urea Transport across Toad Urinary Bladder by Vasopressin: Effect of Periodate Oxidation of the Mucosal Cell Surface

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Summary. Oxidation of toad urinary bladder epithelial cell membranes by periodate in the bathing medium altered vasopressin-stimulated transport of urea or of water and sodium depending on whether periodate bathed the mucosal or serosal surface. Subsequent reduction with tritiated borohydride was then used to label the oxidized cell membrane components. I found that:

1. Vasopressin or adenosine-3′,5′-monophosphate-stimulated urea transport was inhibited after bladders had been exposed to $NaIO_4$ in the *mucosal* medium. Basal rates of urea flow and both basal and hormone-stimulated rates of sodium and water flux remained unaltered.

2. $NaBH_4$ reduction of the oxidized tissue did not restore transport toward normal. Periodate treatment of the mucosal surface before borotritiide reduction increased tritium incorporation into epithelial cells 35-fold, principally into two membrane-associated polypeptides.

3. The response to periodate in the serosal medium was entirely different from that to periodate in the mucosal solution. Periodate in the serosal bath inhibited both basal and hormone-stimulated short-circuit current and vasopressin-stimulated osmotic water flow. Subsequent reduction with borotritiide, however, only increased tritium incorporation two-fold.

Thus, oxidation by periodate separates a vasopressin-stimulated transport system for urea from those for water and sodium, and allows labelling of several membrane glycoproteins which may be associated with the urea transport pathway.

Vasopressin (AVP) affects the toad urinary bladder and mammalian medullary collecting duct similarly [15]. Binding of the hormone at the serosal surface of epithelial cells changes the structure of the mucosal surface, increasing its permeability to water, sodium, and, at least in the toad, to urea and small solutes. Water and solutes traverse the membrane by separate pathways [21, 22, 37]. The luminal plasma membrane of the bladder epithelial cells possesses a vasopressin-responsive, amide-specific, saturable pathway [23] apparently involving a membrane

protein [37]. Amide transport has been selectively inhibited at the luminal membrane by several agents, including phloretin [21], tannic acid, and chromate [37], but no specific sites of action for these agents have been demonstrated.

Membrane components responsible for a specific physiological function, such as urea transport, could be identified and characterized if the chemical treatments that alter function also allow labelling of affected molecules. I now report the effects of periodate oxidation on transport properties of toad bladder epithelial cells. Sites modified by treatment with periodate were subsequently labelled by reduction with tritiated borohydride.

Periodate oxidatively cleaves 1,2-dihydroxy groups to dialdehydes [5, 27] and has been used extensively in structural analysis of carbohydrates of both proteins and lipids. Oxidation by periodate of plasma membranes of lymphocytes [34, 35, 42] and erythrocytes [19] alters their properties. Oxidation by periodate followed by reduction by borotritiide has been used to label membrane glycoproteins and glycolipids [9, 24]. Since the carbohydrate residues of glycoproteins and glycolipids probably reside on the external face of the plasma membrane [16, 31], it has been proposed that all the effects of periodate and borotritiide arise from modification of external saccharide residues, principally those of sialic acid [24]. In the studies to be described I found that periodate treatment of luminal and serosal surfaces of the bladder affected vasopressin-stimulated transport differently. Periodate inclusion in the luminal bath blocked vasopressin-induced urea flux while its inclusion in the serosal bath blocked osmotic water and sodium fluxes. The inhibition of vasopressin-stimulated urea transport produced by periodate oxidation of the luminal surface was not reversed by subsequent borohydride reduction. Reduction with borotritiide labelled at least two membrane components.

Materials and Methods

Female Dominican toads (Bufo marinus) were obtained from National Reagents (Bridgeport, Conn.) and kept at room temperature on damp peat moss. Toads were double-pithed and urinary hemibladders were excised, tied to glass tubes and washed 3 times inside and out with amphibian phosphate buffered Ringer's solution (110 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$, 5 mM sodium phosphate, pH 7.4, 230 mOsm), and finally filled as described below. In all experiments, the serosal medium was aerated and either solutions bathing both serosal and mucosal surfaces were stirred with magnetic bars or hemibladders were shaken in a Dubnoff shaker. Paired control bladders were treated exactly like the

experimental bladders, but the modifying agent was omitted. All transport studies were performed at room temperature.

Tritiated sodium borohydride (specific activity 110–330 mCi/mmole) and ^{14}C-urea (specific activity 2 mCi/mmole) were obtained from New England Nuclear (Boston, Mass.). Vasopressin was either Pitressin from Parke-Davis (S. Hackensack, N.J.), or arginine-vasopressin from Sigma (St. Louis, Mo.), and 8-bromo-adenosine-3′,5′-monophosphate (8-Br-cAMP), an analogue of adenosine-3′,5′-monophosphate, was obtained from Sigma or Plenum Chemicals (Hackensack, N.J.). Triton X-100 (Ruger Chemical Co., Brooklyn, N.Y.), toluene (Fisher) and Omnifluor (New England Nuclear) were mixed in the proportions 3 vol/7 vol/4 g/liter [35] for liquid scintillation counting. Collagenase and hyaluronidase were obtained from Worthington Biochemical (Freehold, N.J.), and purified pancreatic RNAse and DNAse and T_1 RNAse from Dr. Gladys Monroy of Albert Einstein College of Medicine. All other chemicals were reagent grade.

Oxidation and Reduction of Membrane Components

Sodium metaperiodate solutions were prepared immediately before use. To study oxidation of the mucosal surface by periodate, bladders were filled with half-strength Ringer's solution containing periodate and immersed in full-strength Ringer's for 10 min at room temperature. To end the reaction, bladders were quickly emptied and filled 4 times with fresh Ringer's and then washed 3 times (10 min each) inside and out, or incubated with 20 mM glucose for 5 min to reduce excess periodate and then washed. Periodate oxidation of the serosal surface was carried out by immersing the Ringer's-filled bladder in Ringer's solution containing periodate for 10 min at room temperature and the reaction stopped by repeated washing. Shorter incubations had proportionally less effect.

In a separate series of experiments, sodium borohydride reduction was performed on hemibladders following periodate oxidation. After periodate oxidation as outlined above, hemibladders were filled with fresh solutions of 20 mM $NaBH_4$ in Ringer's, final pH 8.9, and immersed in a serosal bath of Ringer's for 20 min at room temperature. Obvious hydrogen evolution ceased after about 15 min. The reaction was stopped by repeated washing. Reduction with sodium borotritiide was accomplished using total $NaBH_4$ concentrations between 3 and 18 mM. Bladders were then washed several times inside and out with Ringer's to remove unreacted $NaBT_4$ and tritiated water. Cells or crude membranes were dialyzed at 0° against 4 to 6 changes of 100 volumes of water for 16 hr to remove noncovalently bound ^{3}H.

Permeability Studies

Hydraulic water permeability and urea transport were measured with bladders filled with half-strength Ringer's containing 1.6 μCi of ^{14}C-urea and immersed in aerated Ringer's [3, 26]. After a 15-min equilibration, baseline water flow was measured gravimetrically during one 15-min interval. Vasopressin or 8-Br-cAMP was then added to the serosal medium at a final concentration of 71 mU/ml or 1.5 mM and water loss measured after two further successive 15-min intervals. Water flux is expressed as μl/15-min period. At the same time intervals, the mucosal and serosal media were sampled for liquid scintillation counting to determine the rate of transfer of urea out of the bladder, expressed as cm/sec. The rate of water loss or urea transport during the basal period was subtracted from the average of the 2 periods after hormone or 8-Br-cAMP stimulation to give the increment due to stimulation. Paired analysis of the data (expressed as mean ± SEM) was performed, using Student's *t*-test [38] or Wilcoxon's rank test [38] to assess differences.

Short circuit current (SCC), an index of active sodium transport [4, 39] was measured in divided Lucite chambers with a single hemibladder forming the barrier between serosal and mucosal solutions. Hemibladders were equilibrated with Ringer's and then one quarter bladder treated while the other quarter served as control using the same protocol as for the bladder sacs. Short circuit current (μA) and potential difference (mV) across the bladder were monitored before, during, and after oxidation, and after vasopressin addition to the serosal medium.

Determination of Periodate Concentration

The periodate concentration in the mucosal medium of bladder sacs was determined spectrophotometrically at 223 nm at the beginning and end of several incubations [1]. The standard curve was prepared with periodate and iodate in appropriate concentrations. No detectable change in the mucosal solution periodate concentration occurred during the course of the experiment; a 5% change could have been demonstrated.

Preparation of Crude Membranes

Epithelial cells were scraped from the bladder stroma into Ringer's solution with a glass slide, and washed with Ringer's. Cells swollen at 0° for 10 min in 15 vol of fresh, ice-cold 1-mM $NaHCO_3$ were broken with a few strokes of a tight Dounce homogenizer. Cell disruption was monitored by phase microscopy. For some experiments whole cell homogenates were used. In others, nuclei were removed by brief centrifugation (10 sec, $900 \times g$, International PRII). The nuclear pellet was resuspended in 6 vol cold 1-mM $NaHCO_3$ and washed twice. The combined supernatant and washings, containing essentially no nuclei, were centrifuged at $10{,}000 \times g$ for 10 min at 0°. The $10{,}000 \times g$ pellet was called the crude membrane fraction. Approximately 80% of two enzymes characteristic of the luminal surface of the toad bladder, ADPase and 5'-nucleotidase [18, 33], was recovered in the crude membrane fraction. The nuclear fraction contained less than 10% of these activities. To determine whether tritiated material remaining in the supernatant was bound to small membrane vesicles, the $10{,}000 \times g$ supernatant was centrifuged for 1 hr at $100{,}000 \times g$. The final supernatant fraction was dialyzed against water, and the nonmembrane bound, nondialyzable content of tritiated macromolecules determined.

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall [25] using bovine serum albumin as a standard. Particulate fractions were often first dissolved by the addition of sodium dodecyl sulfate (SDS) to 1%.

Content of sialic acid in whole cells was established by the thiobarbituric acid method [41]. Cells were hydrolyzed in 25 mM H_2SO_4 at 80 °C for 1 hr, the neutralized supernatant fraction chromatographed on Dowex 1×8 (formate) and sialic acid eluted with 0.4 N HCOOH.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Whole cells or crude membrane fraction were solubilized with 2% SDS, 2% 2-mercaptoethanol in the presence of an appropriate buffer, by heating for 5 min, 100°. Glycerol containing 40 μg/ml pyronin Y was added to 10% by volume. Gels were prepared according to Fairbanks, Steck and Wallach [10, 11], Neville [30], or Maizel [20]. Electrophoresis was performed at 2 mA/tube until the pyronin Y marker was within 5 mm of the bottom of the gel. Gels were stained with Coomassie Blue or periodic-acid-Schiff stain (PAS) [11] or cut into 1 mm sections which were dissolved in 0.5 ml of 30% H_2O_2 at 50° for 6–16 hr and counted in Triton-Toluene-Omnifluor [35].

Results

Effect of Periodate in the Mucosal Bath on Transport

Urinary bladders were exposed to $NaIO_4$ under various conditions to determine its effects on transport. The vasopressin-stimulated increment in urea transport was inhibited 53% in bladders treated on the luminal surface with 5 mM $NaIO_4$ in half-strength Ringer's for 10 min (Fig. 1). $NaIO_4$, 20 mM, led to 95% inhibition while lower concentrations (0.5 or 2 mM $NaIO_4$) were without effect. Even 20 mM $NaIO_4$ did not significantly decrease AVP-stimulated osmotic water flux (Fig. 1). Basal (nonhormone stimulated) levels of osmotic water flow and urea transport

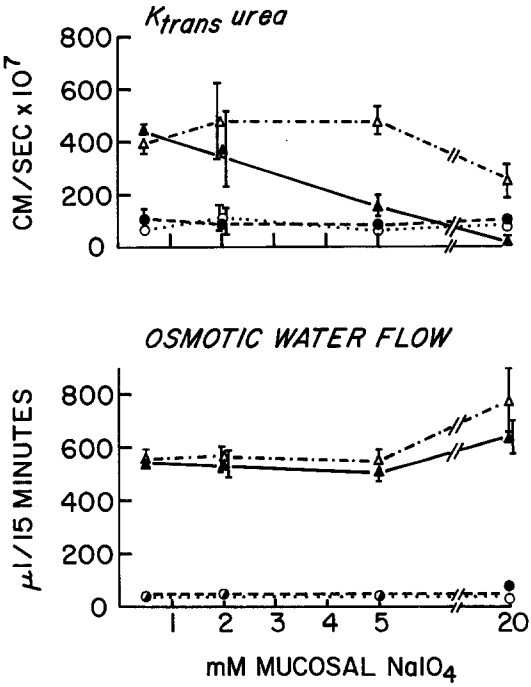

Fig. 1. Basal and AVP-stimulated osmotic water flow and urea transport after periodate oxidation of the mucosal surface. Experimental hemibladder sacs were filled with half-strength phosphate Ringer's with (in mM) 0.5 $NaIO_4$ (pH 7.4), 2 $NaIO_4$ (pH 7.1), 5 $NaIO_4$ (pH 6.95) or 20 $NaIO_4$ (pH 6.6) for 10 min at room temperature, while control hemibladders were incubated with half-strength Ringer's at the stated pH. Variation in pH reflected increasing periodate concentration in the buffer. After washout, osmotic water flow and urea flux were measured before and after AVP addition (71 mU/ml) to the serosal medium. Only AVP-stimulated urea flow was significantly altered (after 5 mM $NaIO_4$, $p < 0.05$; after 20 mM $NaIO_4$, $p < 0.005$). The vertical bars represent mean ± SEM for at least 4 determinations. ··○·· control, basal transport; –●– $NaIO_4$, basal transport; -·-△-·- control, increment after AVP addition; ▲ $NaIO_4$, increment after AVP addition

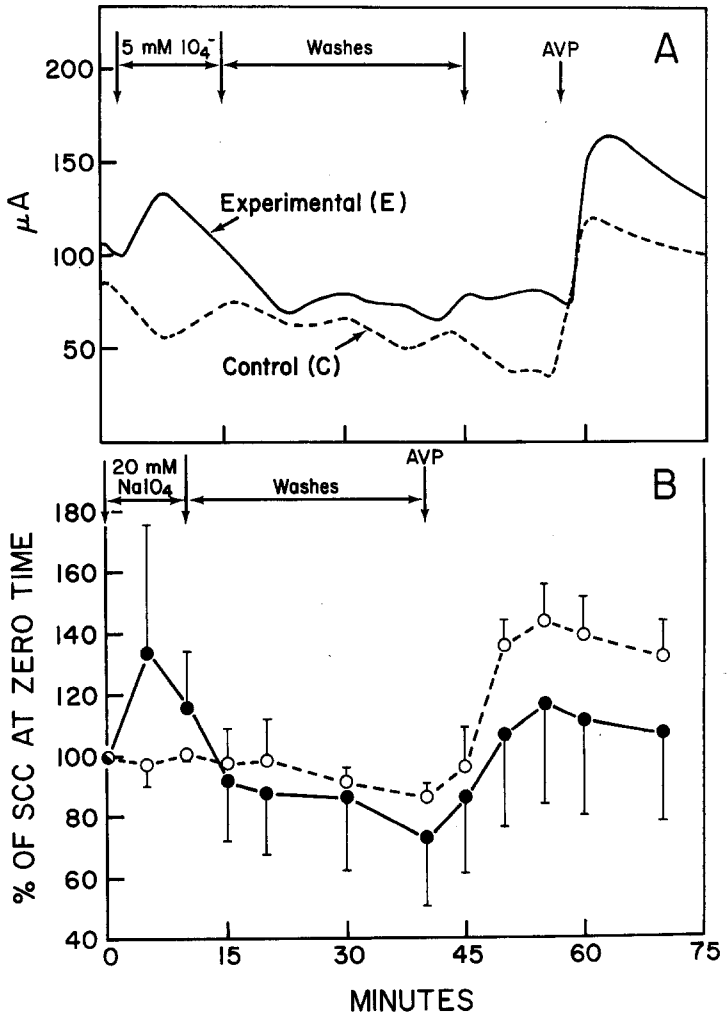

Fig. 2. SCC during periodate oxidation of the mucosal surface, washout, and subsequent AVP stimulation. Experimental quarter bladders in a divided Lucite chamber were treated as for sacs. Short circuit current was continuously monitored (μA); potential difference (mV) across the bladder was measured at intervals and resistance (Ω) calculated as mV/μA. (*A*) Treatment with 5 mM $NaIO_4$, one of five experiments. The baseline potential difference was experimental (*E*), 50 mV and control (*C*), 50 mV; after $NaIO_4$ treatment, *E*, 34 mV and *C*, 24 mV; after washout *E*, 37 mV and *C*, 35 mV; at the peak of the response to addition of 71 mU AVP/ml serosal medium, *E*, 55 mV and *C*, 58 mV. The baseline resistance, $E=485\,\Omega$ and $C=586\,\Omega$; after $NaIO_4$ treatment, $E=378\,\Omega$ and $C=480\,\Omega$; after washout, $E=378\,\Omega$ and $C=480\,\Omega$; and at the peak of the AVP response, $E=335\,\Omega$ and $C=464\,\Omega$. (*B*) Incubation with 20 mM $NaIO_4$, mean ± SEM of 4 experiments. ○, control; ●, experimental

Table 1. AVP-stimulated K_{trans} urea and osmotic water flow after $NaIO_4$ treatment

	K_{trans} ^{14}C urea, cm/sec $\times 10^7$			Osmotic water flow, μl/15 min		
	C	E	Δ(C-E)	C	E	Δ(C-E)
Incubation with 5 mM $NaIO_4$ in the MUCOSAL medium						
Control, buffer End reaction by washing	412±26	200±55	−211+66 (6)[a]	546±44	510±39	−36±38 (6)
Control, buffer End reaction with 20 mM glucose	402±32	233±64	−169±51 (10)[b]	518±42	504±49	−15±28 (9)
Both hemibladders, $NaIO_4$ C, end reaction with glucose E, end reaction by washing	250±115	269±132	+29±16 (4)	449±41	415±41	−34±36 (4)
Both hemibladders, $NaIO_4$ C, add glucose *with* $NaIO_4$ E, end reaction with glucose	328±90	110±30	−227±96 (8)[c]	421±50	367±77	−52±22 (7)
Incubation with 5 mM $NaIO_4$ in the SEROSAL medium						
Control, buffer End reaction by washing	430±61	385±69	−44±24 (6)	415±52	139±36	−275±44 (6)[d]

Hemibladders were exposed to periodate in halfstrength Ringer's on the mucosal surface, or in full strength Ringer's on the serosal surface, for 10 min at 23°. After periodate had been removed by washing or reduced with glucose, both surfaces were washed three times, and basal and hormone-stimulated urea and water fluxes measured. *C*, control; *E*, experimental. Number of experiments shown in parentheses.

[a] $p < 0.05$ by student's *t*-test.

[b] $p < 0.01$ by student's *t*-test.

[c] $p < 0.05$ by Wilcoxon's signed rank test for pair differences.

[d] $p < 0.005$ by Student's *t*-test.

were unaltered (Fig. 1). Vasopressin elicited normal increases in short circuit current and potential differences across the bladder after $NaIO_4$ treatment of the mucosal surface; Fig. 2*A* shows a typical experiment with 5 mM $NaIO_4$ and Fig. 2*B* the mean ± SEM of 4 experiments with 20 mM $NaIO_4$ expressed as % of SCC before treatment. Thus, limited exposure of the luminal surface to $NaIO_4$ alters only vasopressin-stimulated urea transport, leaving water flow and sodium transport unchanged.

In early experiments, oxidation by periodate was ended by rapid addition of glucose to a final concentration of 20 mM (Table 1). However, dialdehyde products of glucose oxidation could conceivably crosslink membrane components and alter transport. Since vasopressin-stimulated urea transport was inhibited to the same extent when periodate oxidation

was followed by rapid washing of the mucosal surface (Table 1), as when the reaction was ended with glucose, all subsequent experiments were terminated by washing. Crosslinking by products of glucose oxidation was also shown to be unimportant in experiments where hormone-stimulated urea transport was much higher in bladders exposed simultaneously to 5 mM $NaIO_4$ and 20 mM glucose than in bladders treated with 5 mM $NaIO_4$ alone (Table 1).

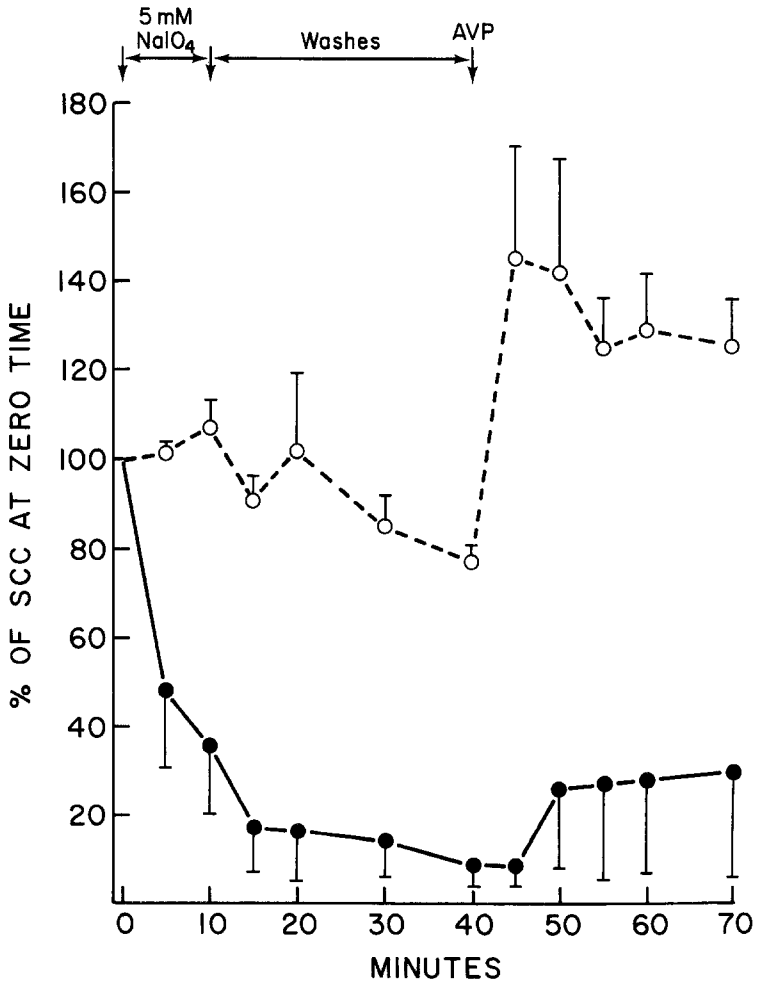

Fig. 3. SCC during oxidation by 5 mM $NaIO_4$ on serosal surface, washout, and subsequent vasopressin stimulation. Experimental quarter bladders were exposed to periodate in the serosal medium as for sacs. Mean ± SEM for 4 hemibladders from different toads, n=4. ○, control. ●, experimental

Effects of Periodate in the Serosal Bath on Transport

Periodate at the serosal surface produced a different pattern of inhibition (Table 1, Fig. 3). Vasopressin-stimulated osmotic water flow was inhibited by 67%, while hormone-stimulated urea flux was consistently slightly, though not significantly, decreased. Basal urea and water flows were not significantly elevated. The potential difference across the bladder fell by 65 ± 10 mv ($p < 0.005$, $n = 6$) during this 10-min incubation. Periodate in the serosal medium caused a rapid drop in resting short circuit current and a greatly decreased response to vasopressin (Fig. 3). Since even 20 mM $NaIO_4$ in the mucosal bath did not affect SCC (Fig. 2*B*), these experiments suggest that little, if any, periodate from the medium bathing the mucosal surface reaches the serosal surface of the cell.

Response to 8-Br-cAMP after Periodate Treatment

8-Br-cAMP was added to the serosal bath instead of AVP to determine whether the mucosal or serosal incubation with periodate affected hormone-receptor interaction and adenylate cyclase activation. The nucleotide did not override inhibition of AVP-stimulated urea transport produced by $NaIO_4$ in the mucosal bath (*compare* Tables 1 and 2), nor did it alter the inhibition of AVP-stimulated osmotic water flow produced by $NaIO_4$ on the serosal surface (Tables 1 and 2).

Table 2. K_{trans} urea and osmotic water flow elicited by 1.5 mM 8-Br-cAMP after periodate treatment

Treatment	K_{trans} urea, cm/sec $\times 10^7$			Osmotic water flow, μl/15 min		
	C	E	Δ	C	E	Δ
Mucosal 5 mM $NaIO_4$	308 ± 54	130 ± 47	-157 ± 36 (6)[a]	358 ± 38	352 ± 39	-6 ± 30 (6)
Serosal 5 mM $NaIO_4$	482 ± 112	377 ± 71	-105 ± 77 (4)	581 ± 56	200 ± 69	-364 ± 77 (4)[b]

After exposure to periodate, hemibladders were washed three times and basal urea and water fluxes determined. Serosal baths were replaced with baths containing 1.5 mM 8-Br-cAMP, and sampled after 1,15, and 30 min for calculation of K_{trans} urea. *C*, control; *E*, experimental. Number of experiments shown in parentheses.

[a] $p < 0.02$.

[b] $p < 0.01$.

Dependence of Transport Alternation on pH of Periodate Oxidation of the Mucosal Surface

Since periodate oxidation of model compounds is a function of pH [5], the dependence of AVP-stimulated urea flux on the pH of oxidation was determined (Fig. 4). Both control and experimental hemibladders were exposed to Ringer's for 10 min at the pH noted. Transport measurements in all experiments were made at pH 7.4 in phosphate-buffered amphibian Ringer's. After oxidation with 5 mM $NaIO_4$ at pH 6.95, the vasopressin increment in urea transport was decreased by 53% (Figs. 1 and 4, Table 1); at pH 6.45, the inhibition was 97% (Fig. 4), and AVP-induced osmotic water flow, not shown, was inhibited 16% ($p < 0.02$). Exposure to 5 mM $NaIO_4$ at pH 7.45 inhibited vasopressin-stimulated urea transport by 54% without altering water flow. Treatment with 2 mM $NaIO_4$ at pH 7.2 was without significant effect (Figs. 1 and 4); at pH 6.45, it brought about a 25% inhibition of urea transport, and at pH 5.95, 47% inhibition without inhibiting AVP-induced osmotic water flux.

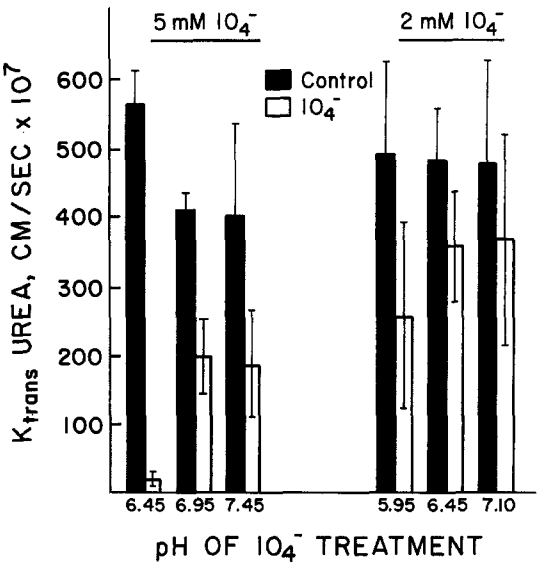

Fig. 4. Hormone-stimulated urea transport after $NaIO_4$ oxidation at different pH's. Both control and experimental hemibladders were incubated with half-strength phosphate Ringer's at the appropriate pH for 10 min; the experimental mucosal solution also contained 2 or 5 mM $NaIO_4$. Subsequent basal and AVP-stimulated osmotic water flows and basal urea transport were similar for control and experimental hemibladders. Inhibition of AVP-induced urea transport was statistically significant with 5 mM $NaIO_4$ at pH 7.45 ($p < 0.02$, n=6), 6.95 ($p < 0.05$, n=6), and 6.45 ($p < 0.01$, n=4) and with 2 mM $NaIO_4$ at pH 6.45 ($p < 0.05$, n=4) and pH 5.95 ($p < 0.02$, n=4)

Attempts to Alter the Action of Mucosal Periodate

Neither urea, the substrate, nor phloretin, a reversible inhibitor of urea transport in toad bladder [21], blocked the action of periodate on transport (Table 3).

To examine the possibility that crosslinks between aldehydes produced by periodate and membrane amino groups were involved in the inhibition of transport, oxidation was carried out in media containing 1 mM pyridoxamine phosphate, 5 mM glycylglycine, or 0.5 mM lysine as competing sources of amino groups which might prevent such crosslinking. None

Table 3. Effect of various agents on the action of 5 mM periodate in the mucosal medium

	AVP	K_{trans} urea, cm/sec $\times 10^7$			Osmotic water flow, µl/15 min		
		C	E	Δ(C-E)	C	E	Δ(C-E)
Control (*C*) and experimental (E), 10^{-4} M phloretin	0	120±19	158±40	62±26 (6)[a]	19±5	40±6	10±4 (6)[a]
E, 5 mM $NaIO_4$	+	612±83	295±83	−350±120 (6)[a]	505±103	548±40	33±24 (6)
C and *E*, 5 mM $NaIO_4$	0	119±61	124±48	5±16 (4)	35±10	31±8	−4±12 (4)
E, 10 mM urea	+	177±30	215±65	38±43 (4)	386±31	460±79	74±38 (4)
C and *E*, Ringer's with 5 mM glycylglycine	0	67±39	51±20	−16±32 (8)	27±5	33±2	3±6 (4)
E, $NaIO_4$	+	318±34	172±39	−147±49 (8)[b]	510±28	507±30	−5±55 (4)
C and *E*, 1 mM pyridoxamine phosphate	0	256±92	198±63	−48±55 (4)	12±5	36±3	5±7 (4)
E, $NaIO_4$	+	466±39	156±95	−310±77 (4)[a]	579±44	523±48	−57±26 (4)
C and *E*, 0.5 mM lysine *E*, $NaIO_4$	+	335±49	150±60	−188±79 (6)[c]	447±35	423±44	−24±25 (6)
C and *E*, 71 mU AVP/ml serosal medium for 15 min; 10 min more with $NaIO_4$	0	198±15	158±27	−39±14 (4)	60±2	47±8	−13±9 (4)
E, 10 mM urea	+	120±31	107±27	−13±12 (4)	342±42	382±34	41±40 (4)
C and *E*, $NaIO_4$	0	208±117	167±85	−42±43 (4)	32±11	42±6	−9±15 (4)
E, reduced with 25 mM $NaBH_4$	+	261±67	258±81	−18±33 (4)	381±54	426±46	45±45 (4)

Phloretin, urea, glycylglycine, etc., were added to the mucosal medium; AVP was added to the serosal bath, and incubation with $NaBH_4$ in the mucosal solution followed the ending of the periodate reaction. Exposure of the mucosal surface to $NaBH_4$ without prior treatment with $NaIO_4$ was without effect (data not shown). 0, basal transport levels, +, increment after addition of 71 mU AVP/ml serosal bath. Number of experiments in parentheses.
[a] $p<0.05$. [b] $p<0.02$. [c] $p<0.01$.

of these compounds are oxidized by periodate. As seen in Table 3, none altered the ability of periodate to inhibit vasopressin-stimulated urea transport. Oxidation by 5 mM periodate had the same effect whether carried out in phosphate-buffered Ringer's (pH 6.95) or in glycylglycine-buffered Ringer's (pH 6.95) (Table 3). Higher concentrations of glycylglycine in the mucosal medium (data not shown) also did not block inhibition by periodate. Thus, if crosslinking were responsible for decreased urea transport, these agents have not blocked it.

Borohydride reduction during the first 6 hours after oxidation of lymphocytes reverses the transformation by periodate [36]. However, treatment of toad bladder with a fivefold molar excess of sodium borohydride after exposure to periodate (Table 3) did not alter periodate inhibition of vasopressin-stimulated urea transport.

Effect of Periodate in Bladders Pretreated with Vasopressin

Vasopressin binding at the serosal surface might change the position or shape of luminal membrane components, thus altering their susceptibility to periodate oxidation. Accordingly, hemibladders were first exposed to vasopressin in the serosal bath for 15 min, and then periodate was added to the mucosal solution. After washout of both periodate and vasopressin, permeabilities were measured for a basal and then two vasopressin-stimulated 15-min periods. Periodate inhibition of subsequent vasopressin-stimulated urea transport (Fig. 5) was more pronounced at periodate concentrations from 2 to 20 mM than in previous experiments in which hemibladders had not been subjected to vasopressin during periodate oxidation (Fig. 1). In fact, after incubation with 5 or 20 mM periodate in the presence of vasopressin, the hormone did not stimulate urea transfer while stimulation of osmotic water flow was significantly decreased. Vasopressin caused similar increases in potential difference in experimental and control bladder sacs.

Treatment with $NaIO_4$ (0.5 to 20 mM) in the presence of AVP led to a constant elevated level of basal urea transfer of about 200×10^{-7} cm sec^{-1}, even at $NaIO_4$ concentrations which had no other apparent effects. The hormone-stimulated increment in urea transport progressively decreased with increasing periodate concentration, even in the presence of considerable osmotic water flow in the direction which might be expected to increase urea flux. Elevated basal urea transport was probably not attributable to "leakiness" or nonspecific permeability after the experimental treatment because (^{14}C)-sucrose flux in the same direction

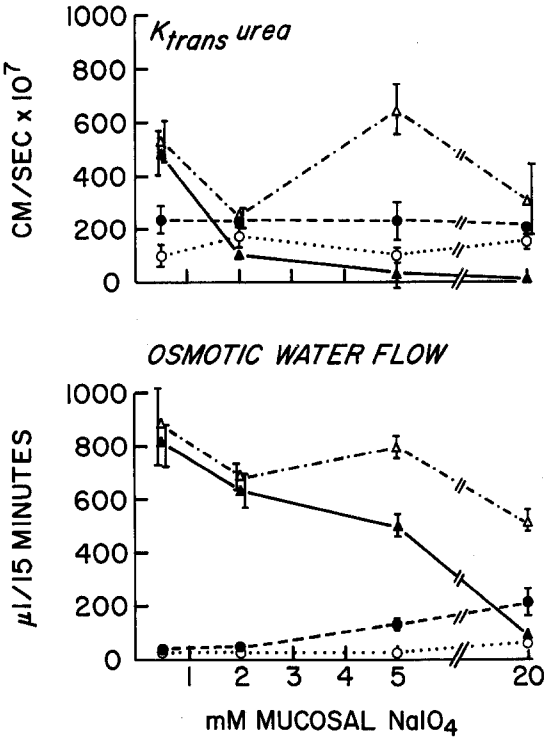

Fig. 5. $NaIO_4$ effect after pretreatment with AVP. Control and experimental bladder sacs containing half-strength Ringer's solution were incubated for 25 min with 71 mU AVP per ml serosal Ringer's. For the last 10 min, the control mucosal medium was replaced with fresh half-strength Ringer's, and the experimental with half-strength Ringer's containing $NaIO_4$. After both surfaces of the hemibladders had been washed for 30 min, transport was measured first without and then with 71 mU AVP/ml serosal bath. At least 4 toads were used for each $NaIO_4$ concentration, and the low level of urea transport for the controls for 2 mM $NaIO_4$ reflects variability among toads. ··○·· control basal; --●-- $NaIO_4$ basal; -·-△-·- control, increment after AVP; ▲ $NaIO_4$, increment after AVP

was not enhanced, and the basal potential difference across the bladder as well as the increased potential difference evoked by vasopressin were comparable to controls.

Urea did not protect the transfer site even during periodate treatment in the presence of vasopressin (Table 3).

Labelling of Membranes with Tritium after Periodate Oxidation

In order to identify the sites altered by periodate, oxidized cells were subjected to borotritiide reduction. After cells were scraped from the stroma, experimental hemibladder epithelial cells averaged

$3.7 \pm 0.7 \times 10^5$ cpm, or 16.8 ± 3.0 nmoles ^3H per hemibladder ($n=4$), while their paired controls, which had not been periodate treated, incorporated $0.08 \pm 0.04 \times 10^5$ cpm, or 0.5 ± 0.2 nmoles ^3H per hemibladder, only 2.5% as much.

Similar experiments with another batch of borotritiide led to incorporation of 16.5 ± 0.8 nmoles ^3H per hemibladder ($n=6$) after treatment with 5 mM $NaIO_4$ and 2.0 ± 0.9 nmoles ^3H per hemibladder in unpaired controls ($n=5$) treated with 0.5 mM $NaIO_4$, a concentration which did not alter transport measurably.

In 15 experiments, an average of 10.6 ± 1.4 nmoles of ^3H was incorporated per mg of protein. Since periodate oxidation and borotritiide reduction of human erythrocytes leads to incorporation of most of the tritium into products of sialic acid [24], toad bladder epithelial cells were subjected to mild acid hydrolysis and their total content of sialic acid established to be 12.7 ± 0.9 nmoles of sialic acid per hemibladder ($n=8$), or 4.3 ± 0.7 nmoles sialic acid per mg protein. Clearly, in toad bladder less than half of the total tritium could be incorporated into products of sialic acid. Other studies (Meryl S. Rubin, *in preparation*) have shown that less than 20% of the incorporated ^3H results from sialic acid alteration.

Tritium crossed the bladder with a permeability of about 400×10^{-7} cm/sec during the last 10 min of the 20-min incubation. Such rapid passage in the absence of AVP is unusual. Two experiments were performed to ascertain whether the ^3H was in borotritiide, borate, or water (by exchange). Only 0.5% of the tritium could not be volatilized by repeated evaporation of serosal baths to remove tritiated water. No radioactivity was lost upon subsequent evaporation from dry methanol to remove boric acid as trimethylborate. To determine whether the tritiated molecule was still a reducing agent, serosal baths were brought to pH 10 with NaOH, and 5 μmoles of mannose added. After 20 hr incubation at 0°, these solutions were also repeatedly evaporated. Again, only 0.5% of the ^3H remained. Thus, the ^3H which moves from the mucosal to serosal baths appears to be mostly ^3H_2O.

Tritium was incorporated into membrane glycoproteins and glycolipids. 82% of the label was in the crude membrane and nuclear pellets and only $18 \pm 2\%$ ($n=11$) in the $10{,}000 \times g$ supernatant. Only $6 \pm 1\%$ ($n=5$) of the tritium remained in the supernatant after centrifugation at $100{,}000 \times g$ for 1 hr and was clearly not membrane bound.

Extraction of cells or crude membranes with 20 vol of cold chloroform/methanol (2:1, v/v) followed by washing of the extract with 0.2 vol

cold water [12] led to recovery of $20 \pm 3\%$ of the tritium in the chloroform layer ($n=8$). The 15–23% of tritium that moved slightly more slowly than the dye front in polyacrylamide gel electrophoresis probably contained not only phospholipids and glycolipids but also small peptides and tritiated borohydride, borate, and H_2O. Pronase solubilized 79–99% of the tritium of crude membranes; in delipidated samples, 99% of the radioactivity was recovered in the $10{,}000 \times g$ supernatant fraction after hydrolysis by pronase.

Labelling after Periodate Oxidation of the Serosal Surface

Treatment with $NaIO_4$ in the serosal bath elicited much less tritium incorporation into crude membranes, at best only twice the level in nonperiodate treated bladders: experimental 0.17 ± 0.14 nmoles ^3H and control, 0.10 ± 0.05 nmoles ^3H per hemibladder ($n=4$).

Labelling after Simultaneous Exposure to AVP and Periodate

The presence of AVP in the serosal bath during exposure of the mucosal cell surface to $NaIO_4$ did not alter tritium incorporation into macromolecules following borotritiide reduction: After periodate alone 12.9 ± 5.1 nmoles ^3H; after AVP and periodate 11.4 ± 2.7 nmoles ^3H per hemibladder ($n=7$ pairs).

Gel Electrophoresis after Periodate Oxidation and Borotritiide Reduction

Crude membrane proteins were resolved by SDS-polyacrylamide gel electrophoresis in several systems. Figure 6 shows a densitometer tracing of Coomassie blue stained proteins superimposed on the ^3H pattern obtained after slicing and dissolution. Crude membranes had similar protein patterns whether prepared from cells exposed to periodate and then borotritiide, or only to borotritiide (not shown). No ^3H peaks were observed on gels of membranes from cells treated only with borotritiide. In the continuous buffer system [11] (Fig. 6), one component (40 to 70% of total tritium) barely entered the gel while a second (R_f 0.27, 10–20% of total tritium) had an apparent molecular weight of about 100,000 daltons. In stained and destained gels (as in Fig. 6), recovery of tritium never exceeded 40%; often no third peak followed the dye front. In unstained gels, 80 to 100% of the label was recovered, and

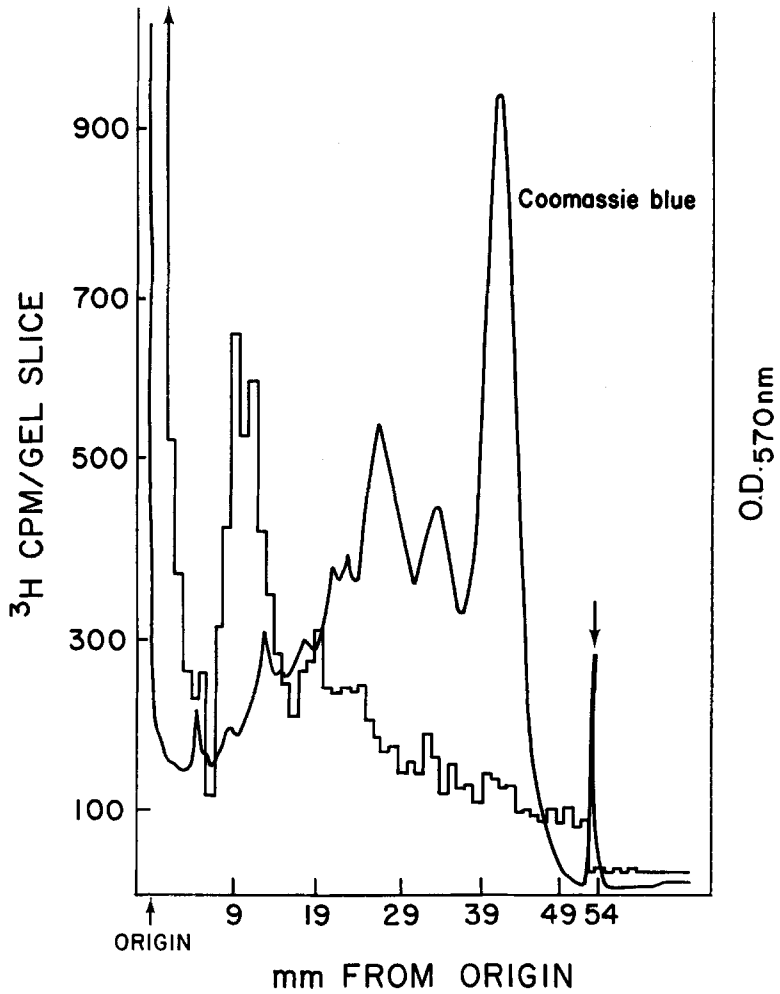

Fig. 6. SDS-plyacrylamide gel electropherogram of crude membranes. Crude membranes were prepared from hemibladders after oxidation by $NaIO_4$ in the mucosal medium and NaB^3H_4 reduction, and subjected to electrophoresis on 5.6% gels (acrylamide/bis ratio 26.7) at 2 mA/gel. Gels were stained with Coomassie blue and destained in 7% acetic acid. The curved trace shows the O.D. $_{570\,nm}$ on a Gilford 2500 linear scanner. The gel was then sliced into 1 mm segments, dissolved in 30% H_2O_2 and ^3H assessed by liquid scintillation counting (box trace). The first two slices contained 9160 and 1170 cpm. The dye front is indicated by an arrow. Gels sliced without staining show a third ^3H peak extending over the 10 slices following the dye front

15 to 23% migrated in a peak slightly slower than the dye front. Presumably acyclic acetals formed by periodate oxidation were hydrolyzed by acetic acid during staining and destaining and the label released from the gel.

The material in the first 6 mm of the gel was in fact within the gel, as rinsing the gel top before removing it from the tube did not remove any radioactivity. These counts might have been in collagen from the bladder stroma, in glycosaminoglycans from the cell surface, in proteins crosslinked by periodate and borotritiide treatment, or perhaps in proteins prevented from migrating by formation of a nucleic acid gel. Accordingly, crude membranes containing about 100 μg protein were treated with 25 μg collagenase in 50 mM Tris chloride, pH 7.4, 5 mM $CaCl_2$ or 2 μg hyaluronidase in 40 mM Tris acetate, pH 5.5 for up to 16 hr at 37° in the presence of 0.4 mM phenylmethyl-sulfonyl fluoride to inhibit serine proteases. Neither an increase in soluble (or dialyzable) tritium nor appreciable change in the proportion of tritium within the first 6 mm of gel was noted. Treatment with a mixture of T_1 RNAse and pancreatic RNAse and DNAse was also without effect. After treatment of crude membranes with pronase (10 μg for 1 hr at 37°), much of the initial peak was degraded to smaller molecules. Crosslinked protein, but not native collagen, would be susceptible to pronase.

Conditions which might allow further characterization of the very large material were sought: electrophoresis at acid pH [10], in discontinuous alkaline buffer systems [20, 30] or with increased gel pore size [28] did not alter the proportion of material in the first 6 mm of gel, consistent with a very large polypeptide.

Although the presence of amino acids during periodate oxidation and borohydride reduction had no effect on inhibition of AVP-dependent urea transport, such compounds might have blocked crosslinking of membrane proteins. In three experiments examining the effect of 0.5 mM glycylglycine (a large excess over the total ^3H incorporated after periodate oxidation), a small decrease in the proportion of ^3H in slices 1–6 to total ^3H on the gel was noted: $68 \pm 1\%$ in controls, and $61 \pm 1\%$ in the presence of glycylglycine, suggesting that glycylglycine did block crosslinking of membrane proteins to some extent.

Alcian blue [40] is a general stain for the saccharide moieties of glycoproteins and glycolipids. PAS [11] stains sialoglycoconjugates principally. Staining by either method requires that a substantial part of the glycoconjugate be carbohydrate. PAS [11] or Alcian Blue [40] staining of gels with as much as 400 μg of crude membrane protein led only to very faint staining on either side of an opaque lipid band (not shown) which corresponded to edges of the label near the dye front. Clearly toad bladder is not rich in sialoglycoproteins.

Discussion

Increased transport of water, sodium, and amides across the epithelial cell of toad urinary bladder in response to vasopressin appears to involve major alteration of its luminal plasma membrane [6, 7, 13, 17]. None of the agents that have been shown previously to specifically inhibit one or another transport phenomenon, however, has allowed further definition of the membrane component(s) affected. Such definition requires use of an agent that either binds very tightly to the membrane or covalently alters it in a unique manner while modifying a specific function; the agent should also provide means of labelling the altered sites. The findings presented here show that sodium metaperiodate is such an agent. Its use in the medium bathing the mucosal surface of the toad bladder diminishes the capacity of the membrane to respond to vasopressin with an increase of urea transport and alters its cell surface to allow subsequent incorporation of tritium from tritiated borohydride. *Two* major components which behave like membrane glycoproteins are labelled by that treatment and may be involved in regulating hormone-sensitive urea transport across the bladder. Although the evidence linking these two phenomena is still inconclusive, it provides a point of departure for future studies further defining the protein and glycoprotein components of the urea transport system.

Several observations suggest that periodate acts at the luminal surface of epithelial cells. Periodate is a relatively large inorganic anion; such anions often traverse membranes slowly [32]. The luminal plasma membrane in the absence of vasopressin is fairly impermeable to hydrophilic solutes, and tight junctions between cells prevent intercellular passage of water and solutes [15]. Furthermore, periodate was used at relatively low concentration in the medium bathing the mucosal surface and was removed after a short period of incubation. Although no gross decrease in the mucosal periodate concentration was demonstrable during a 10-min incubation, transport across the mucosal cells was not ruled out unequivocally. Rigorous proof would require use of $Na^{125}IO_4$ entirely free of $^{125}I^-$; such a reagent is unavailable.

The same concentration of periodate that acted on the mucosal surface to inhibit stimulation of urea transport by vasopressin, depressed vasopressin-dependent water and sodium fluxes when acting on the serosal surface. Even a fourfold higher periodate concentration in the luminal bath (20 mM) did not alter SCC in response to hormone, again suggesting that no appreciable amount of periodate reached the basolateral plasma membrane.

Decreased cyclic AMP formation in response to vasopressin did not appear to be responsible for diminished transport subsequent to oxidation by periodate of either mucosal or serosal components, as addition of 8-Br-cAMP did not override the block.

Some evidence that periodate in the mucosal solution alters cell membranes may be adduced from borotritiide reduction and differential centrifugation of cell components. Within the limits of reliability of cell fractionation procedures, 94% of the label appeared to be incorporated into membranes. The $10{,}000 \times g$ pellet contained 82%, the $10{,}000 \times g$ supernatant only 18%, and the $100{,}000 \times g$ supernatant only 6% of the ^3H incorporated into macromolecules.

Periodate most probably cleaved the carbon-carbon bond between vicinal hydroxyls of carbohydrate residues, leading to formation of acyclic acetals in the carbohydrate chain [5, 27], with aldehydes replacing vicinal hydroxyls. Borohydride or borotritiide would reduce the aldehydes to (tritiated) alcohols [5, 29]. Vasopressin-sensitive urea transport was more strongly inhibited as the pH of oxidation was lowered, so oxidation of possible N-terminal serine or threonine residues is unlikely, as this falls off rapidly at $pH < 7.5$ [5]. Oxidation of other amino acids also decreases as the pH is decreased [5, 8]. Studies which have demonstrated destruction of methionine, tryptophan, cysteine, tyrosine and histidine residues have generally used higher concentrations of oxidant for longer periods, often in the absence of carbohydrate residues [2, 8]. Amino sugars are usually *N*-acetylated *in vivo* [29], and thus more resistant to oxidation by periodate [5].

Macromolecule-bound ^3H near the gel tops appeared despite prior treatment with collagenase, hyaluronidase or nucleases, so probably collagen, certain glycosaminoglycans or nucleic acids are not the targets for reaction with periodate. However, the material was sensitive to short exposure to pronase, suggesting that tritium was incorporated into membrane proteins, more specifically, into carbohydrate residues of membrane glycoproteins (M.S. Rubin and C.F. King, *in preparation*). Polypeptides that either bind little or no SDS and thus are relatively uncharged, or are very large, would remain near the gel top. Two overlapping peaks were obtained after gel chromatography of membranes in denaturing solvents. Upon subsequent analysis by disc gel electrophoresis, fractions from the first peak that emerged from the column were shown to correspond to the material near the top of the gel, and fractions from the second peak contained principally the less rapidly migrating species on electrophoresis (M.S. Rubin and C.F. King, *in preparation*). Thus, the

material at the top of the gel is probably a large polypeptide(s) of very restricted mobility. Such a large species could be generated by crosslinking between separate polypeptide chains, by reaction of membrane amino groups with aldehydes formed by periodate oxidation. In that case the material could be an artifact of the oxidation and subsequent reduction of Schiff bases to secondary amines by borotritiide. Recently, Guidotti [14] suggested that oligomeric glycoproteins that span the plasma membranes of eukaryotic cells transport solutes by undergoing conformational changes; in that case, crosslinking, though artifactual, might indicate some proximity *in situ* between the crosslinked polypeptides. That possibility is being investigated.

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