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The Effect of Tanning Agents on the Permeability of the Toad Bladder to Water and Solutes*

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Summary. Previous studies with phloretin have shown that the movement of urea and other solutes across the toad bladder can be inhibited with no effect on osmotic water flow, active sodium transport, or the movement of ethanol and ethylene glycol. These findings have suggested that a vasopressin-sensitive carrier is involved in the transport of solutes such as urea across the luminal membrane of the epithelial cell. The present paper describes the effect of two agents other than phloretin: tannic acid and chromate, on water and solute movement across the bladder. The pattern of action of these two agents resembles that of phloretin, and supports our earlier findings of the independence of solute and water movement. The effect of chromate on urea movement is seen only in the presence of vasopressin, and only if chromate is added prior to vasopressin. Chromate also proves to be an irreversible inhibitor of urea movement. The implications of these findings are discussed. In view of the known interactions of both agents with proteins, it is suggested that carrier-mediated transport of urea proceeds across a protein component of the membrane.

The movement of urea across cell membranes has been attributed to several mechanisms, including passive diffusion through aqueous channels (Andersen & Ussing, 1957; Leaf & Hays, 1962); facilitated diffusion (Murdaugh, Robin & Hearn, 1964; Hunter, George & Ospina, 1965); and active transport (Forster, 1954; Ullrich, Rumrich & Schmidt-Nielsen, 1967). The variety of models for urea transport reflects the variety of animal species studied, differences in transport mechanisms in cells of a given species, and the particular experimental conditions employed. Evidence for facilitated diffusion has been obtained largely through the use of agents that inhibit urea movement; Hunter, George and Ospina (1965), for example, proposed

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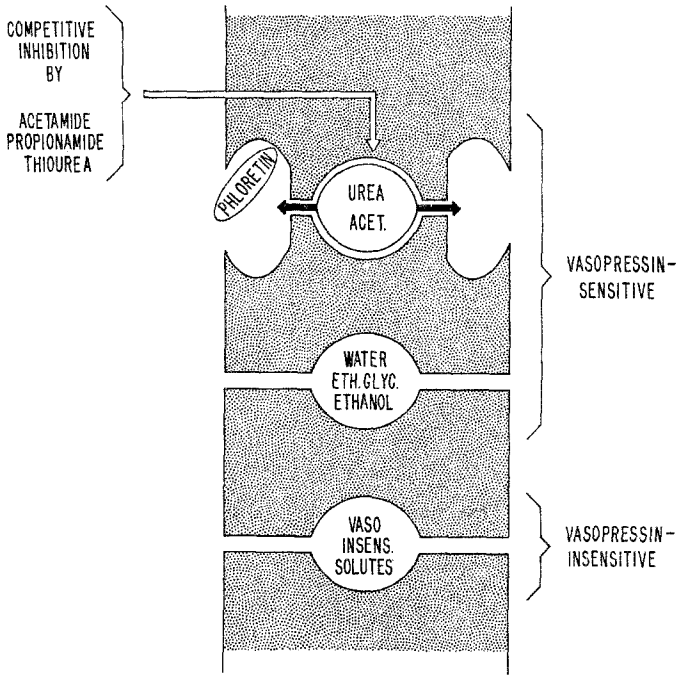

Fig. 1. Schematic view of transport pathways across luminal membrane of toad bladder epithelial cell. The top pathway is a vasopressin-sensitive, phloretin-inhibited pathway for the facilitated diffusion of amides and certain nonamides (including thiourea and formaldehyde). Water, ethylene glycol, and ethanol move via one or more vasopressin-sensitive, phloretin-insensitive pathways (center), and other solutes move via pathways which are insensitive to vasopressin and phloretin (bottom)

that the inhibition of urea movement across the human erythrocyte membrane by tannic acid suggested transport by facilitated diffusion. More recently, Macey and Farmer (1970) reported that phloretin inhibited the movement of urea across the human erythrocyte membrane, but had no effect on osmotic water flow.

We have extended Macey and Farmer's findings to the toad bladder, and have shown that 10^{-4} M phloretin in the luminal bathing medium inhibits the movement of all amides, thiourea, and formaldehyde across the vasopressin-stimulated bladder, but has no effect on osmotic water flow, active sodium transport, or the movement of ethanol and ethylene glycol (Levine, Franki & Hays, 1973*a*). Recent studies have shown that ^{14}C urea and ^{14}C acetamide movement across the toad bladder shows saturation kinetics at high concentrations of unlabeled acetamide (Levine, Franki & Hays, 1973*b*). These findings in the toad bladder indicate that several pathways appear to

be present in the luminal membrane of the epithelial cell: one for osmotic water flow, a second for the carrier-mediated, phloretin-inhibited movement of certain solutes (notably the amides), and possibly a third for solutes such as ethanol and ethylene glycol, whose movement is stimulated by vasopressin, but not affected by phloretin (Fig. 1). Another pathway may transport vasopressin-insensitive solutes.

We wish to report studies showing that two agents other than phloretin, tannic acid and chromate, both of which are used as protein cross-linking agents in the tanning of leather, are capable of modifying solute movement across the toad bladder independent of water flow. The pattern of action of these two agents resembles that of phloretin, but also differs in certain important respects. Our findings support our earlier evidence that water and solute movement occur independently, and suggest that urea movement takes place through a protein component of the cell membrane.

Materials and Methods

The permeability coefficients (K_{trans}) of ^{14}C urea, ethanol, and ethylene glycol were determined from lumen to serosa in paired bladder sacs tied to glass bungs and removed from Dominican toads (*Bufo marinus*, National Reagents, Bridgeport, Conn.). In the tannic acid experiments, control bladders were washed in phosphate-buffered amphibian Ringer's solution (120 mM Na^+, 4.0 mM K^+, 0.5 mM Ca^{++}, 116 mM Cl^-, 1.0 mM $H_2PO_4^-$, 4.0 mM HPO_4^{--}, pH 7.4, 230 mOsm/kg H_2O), filled with 5 ml of Ringer's diluted 1:10 with distilled water, and containing the isotope to be studied, and placed in beakers containing 30 ml of full-strength Ringer's. Paired test bladders were treated in the same fashion, except that the mucosal solution contained 1 or 5×10^{-4} M gall nut tannic acid, kindly supplied as a purified powder by Mr. Richard Boosey, Mallinckrodt Chemical Works, St. Louis, Mo. Control bladders for the chromate experiments were similarly washed in phosphate Ringer's, but during study were filled with 6 ml 1:10 Ringer's maintained at pH 5.0 with 2 mM sodium phthalate. The serosal bathing medium was 35 ml phosphate Ringer's at pH 7.4. The paired test bladders were filled with a mucosal solution at pH 5.0 containing 1 mM sodium dichromate. Under these conditions, $HCrO_4^-$ is the predominant species (Cotton & Wilkinson, 1972). Air was bubbled through the serosal bath and stirring was provided in the outside bathing solution by rotating bar magnets. The inside solution was unstirred, reducing somewhat K_{trans} of the more rapidly moving solutes. Osmotic water flow was determined gravimetrically (Bentley, 1958). Short-circuit current was determined in Lucite chambers with a central dividing partition (Sharp & Leaf, 1964). ^{14}C urea, ethanol, ethylene glycol, and ^{51}Cr were supplied by New England Nuclear Corp., Boston, Mass. Counting was done in a Tri-Carb liquid scintillation counter (Packard Instrument Co., La Grange, Ill.) and, for ^{51}Cr, in an Autogamma scintillation spectrometer (Packard Instrument Co.). In all experiments, results obtained in the test bladder were compared to the control bladder by the method of paired analysis (Snedecor & Cochran, 1967). K_{trans} was determined for one or two consecutive 15-min periods prior to vasopressin, and for two consecutive 15-min periods following vasopressin. Where appropriate, the pre- and postvasopressin periods are shown as single 30-min periods for ease of presentation.

Results

Effect of Tannic Acid on Water and Solute Movement

The effect of 5×10^{-4} M tannic acid in the luminal bathing medium on the movement of water, urea, ethylene glycol, and ethanol is shown in the upper half of Table 1. There was a slight (13%) depression in vasopressin-stimulated water movement, and a marked inhibition of K_{trans} urea both before and after vasopressin. K_{trans} ethanol was not affected by tannic acid; K_{trans} ethylene glycol was unaffected in the absence of vasopressin but significantly depressed following hormone.

A complete dissociation of vasopressin-stimulated water flow and K_{trans} urea could be obtained by reducing the concentration of tannic acid to 1×10^{-4} M (lower half of Table 1). There was no significant effect of tannic acid on osmotic flow following vasopressin. Baseline water flow (determined for one, rather than two, 15-min periods) was, however, significantly decreased compared to control. K_{trans} urea was significantly depressed by approximately 40% both before and after hormone. K_{trans} ethylene glycol was also significantly depressed following vasopressin, as it was with the higher concentration of tannic acid.

The effect of 5×10^{-4} M tannic acid on active sodium transport in four paired bladder halves is shown in Fig. 2. There was a small and insignificant

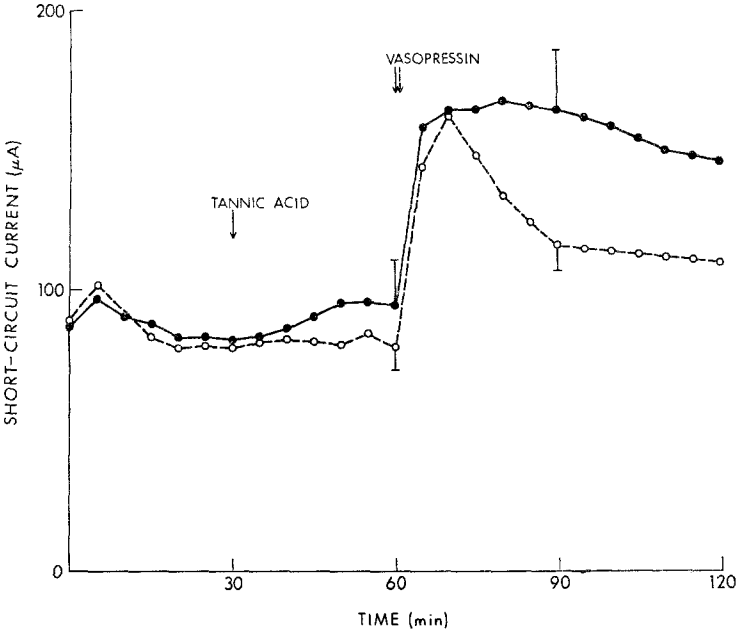

Fig. 2. Effect of tannic acid on short-circuit current of test bladders (closed circles). Control bladders shown with open circles. Four paired experiments; vertical bars ± 1 SEM

Table 1. Effect of tannic acid on permeability of bladder to nonelectrolytes

Compound	Control bladder		Test bladder		Δ (Control-Test); p value	
	I	II	I	II	I	II
5×10^{-4} M tannic acid[a]						
Water[b] (14)[c]	2.3±0.2[e]	36.7±1.8	2.6±0.3	32.0±1.5	−0.3±0.3 (NS)	4.7±1.7 (<0.02)
Urea ^{14}C[d] (6)	148±31	514±51	28±6	135±19	120±25 (<0.01)	379±45 (<0.001)
Ethylene glycol ^{14}C (8)	14.6±1.9	27.5±3.9	14.5±1.2	21.1±1.7	0.1±1.0 (NS)	6.4±2.5 (<0.05)
Ethanol ^{14}C (4)	693±91	937±77	766±101	1071±122	−73±88 (NS)	−134±78 (NS)
1×10^{-4} M tannic acid[f]						
Water (10)	2.4±0.2	36.3±2.9	1.9±0.2	34.2±2.7	0.5±0.2 (<0.05)	2.2±1.6 (NS)
Urea ^{14}C (4)	213±37	461±92	114±30	286±80	99±17 (<0.02)	175±28 (<0.01)
Ethylene glycol ^{14}C (6)	15.0±1.4	28.0±4.4	11.8±0.8	16.6±1.1	3.2±1.5 (NS)	11.3±3.4 (<0.05)

[a] Periods I and II, 30 min; vasopressin added after period I.
[b] In μliter per min per sac in this and following Tables.
[c] Numbers in parentheses indicate number of experiments.
[d] In cm per sec × 10^7 for urea, ethylene glycol, and ethanol, in this and following Tables.
[e] ±1 SEM.
[f] Period I, 15 min; period II, 30 min; vasopressin added after period I.

increase in resting short-circuit current following the addition of tannic acid to the luminal solution of the test chamber. Following vasopressin, the initial response of test and control bladder halves was identical; there was, however, a significant delay in the return of the test bladder toward control values. Thus, short-circuit current at 90 min in the test half was 70 μamps above its 60-min value and, in the control half, only 36 μamps above its 60-min value ($\Delta = 34 \pm 8$ μamps; $p < 0.05$). There was no significant difference in the ohmic resistance between test and control bladders in these four paired experiments.

Addition of Tannic Acid Following Vasopressin

To determine whether the inhibitory effect of tannic acid on vasopressin-stimulated urea movement required the addition of tannic acid prior to the addition of vasopressin, paired bladders were treated identically through one 15-min baseline period and one 15-min vasopressin period. 5×10^{-4} M tannic acid was then added to the luminal medium of the test bladder, and two additional vasopressin periods were carried out. As shown in Fig. 3, there was a sharp and significant ($p < 0.02$) decline in K_{trans} urea following the addition of tannic acid to the test bladder. Therefore, tannic acid inhibited urea movement when added after vasopressin.

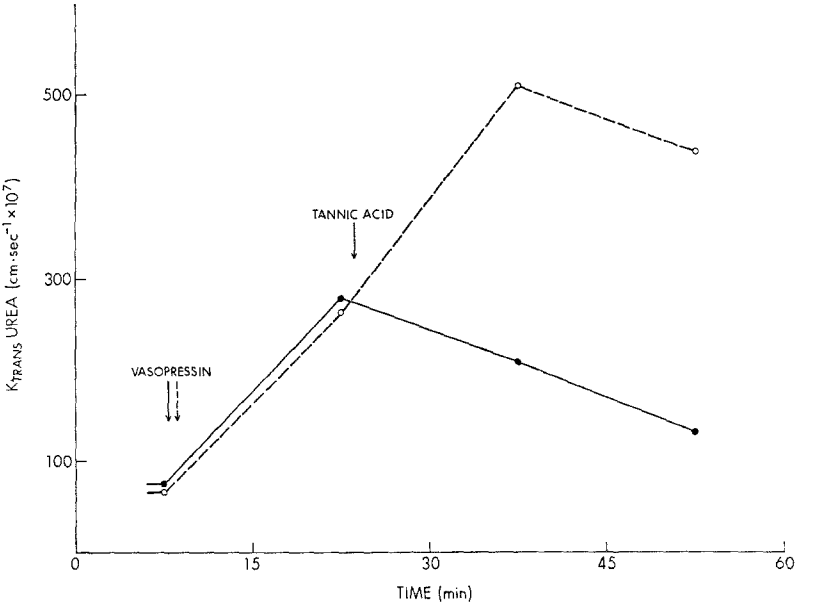

Fig. 3. Effect of addition of tannic acid following vasopressin on K_{trans} urea. Test bladder shown with closed circles; control bladder with open circles. Points are mean values for 4 paired experiments

Reversibility of Effect of Tannic Acid

A series of experiments was carried out to determine whether water and solute movement across bladders exposed to 5×10^{-4} M tannic acid would return to their baseline state after removal of both tannic acid and vasopressin. Following the measurements of water flow and solute movement shown in Table 1, which involved a total exposure time to tannic acid of 70 min in the test bladder, tannic acid was removed from the luminal bathing medium and replaced by 1:10 Ringer's solution. The bladders were also placed in an outside bathing medium free of vasopressin. Fresh inside and outside solutions were introduced 3 times over a period of 30 min, permitting any residual vasopressin effect to disappear. The control bladders were treated in an identical fashion. The results are shown in Table 2 (note that the values for periods I and II for urea are identical to those of Table 1; the values for water include 10 of the 14 experiments shown in Table 1). Following washout of tannic acid and vasopressin (period III), osmotic water flow returned to baseline values. K_{trans} urea, however, rose sharply in period III in the test bladders to a level considerably above period II. K_{trans} ethylene glycol in period III remained at the same level as in period II.

Effect of Chromate on Water and Solute Movement

The effect of the chromate ion on water and solute movement is shown in the upper half of Table 3. In these experiments, the pH of the luminal bathing medium was maintained at 5.0 with phthalate (*see* Materials and Methods). Chromate had no significant effect on osmotic water flow in 12 paired experiments. It markedly inhibited the movement of urea, but, in contrast to tannic acid, only in the presence of vasopressin. Chromate had no effect on K_{trans} ethylene glycol or ethanol. There was no effect on active sodium transport in either the absence or presence of vasopressin, as shown in a representative experiment (Fig. 4). Identical results were obtained in two additional experiments.

When paired sac experiments were conducted at luminal pH 7.4, chromate significantly inhibited vasopressin-stimulated K_{trans} urea. The effect was less marked (36% inhibition) than at pH 5.0 (60% inhibition). Also at pH 7.4, there was a small but significant depression of vasopressin-stimulated water movement (lower half of Table 3).

Addition of Chromate Following Vasopressin

A second basic difference in the action of chromate compared to that of tannic acid was seen in experiments in which chromate was added to the

Table 2. Effect of washout of tannic acid on permeability to nonelectrolytes

Compound	Control bladder			Test bladder			Δ (Control-Test); p value		
	I[a]	II	III	I	II	III	I	II	III
Water (10)	2.3±0.2	35.4±1.8	1.8±0.5	2.6±0.2	30.2±1.7	3.5±0.5	−0.3±0.2 (NS)	5.2±1.8 (<0.05)	−1.7±0.9 (NS)
Urea ^{14}C (6)	148±31	514±51	65±22	28±6	135±19	250±27	120±25 (<0.01)	379±45 (<0.001)	−185±39 (<0.01)
Ethylene glycol ^{14}C (4)	14.3±3.0	31.8±6.4	10.5±2.1	15.3±1.7	22.3±2.4	20.3±1.3	−1.0±1.5 (NS)	9.5±4.3 (NS)	−9.8±1.5 (<0.001)

[a] Periods I, II, and III 30 min each; vasopressin added after period I; tannic acid removed after period II.

Table 3. Effect of 1 mM chromate on bladder permeability to nonelectrolytes

Compound	Control bladder		Test bladder		Δ (Control-Test); p Value	
	I[a]	II	I	II	I	II
pH 5.0						
Water (12)	2.0±0.2	39.3±4.1	1.9±0.3	34.2±2.5	0.1±0.3 (NS)	5.1±2.7 (NS)
Urea ^{14}C (4)	81±62	304±76	89±51	122±59	−8±12 (NS)	182±23 (<0.01)
Ethylene glycol ^{14}C (4)	8±1	20±3	12±4	20±5	−4±4 (NS)	0±4 (NS)
Ethanol ^{14}C (4)	685±29	903±89	736±39	886±16	−51±59 (NS)	17±82 (NS)
pH 7.4						
Water (5)	2.3±0.3	36.2±6.3	3.9±0.6	30.6±5.0	−1.6±0.6 (NS)	5.6±1.9 (<0.05)
Urea (5)	65±16	439±65	109±38	279±69	−44±27 (NS)	160±30 (<0.01)

[a] Period I, 15 min; period II, 30 min; vasopressin added after period I.

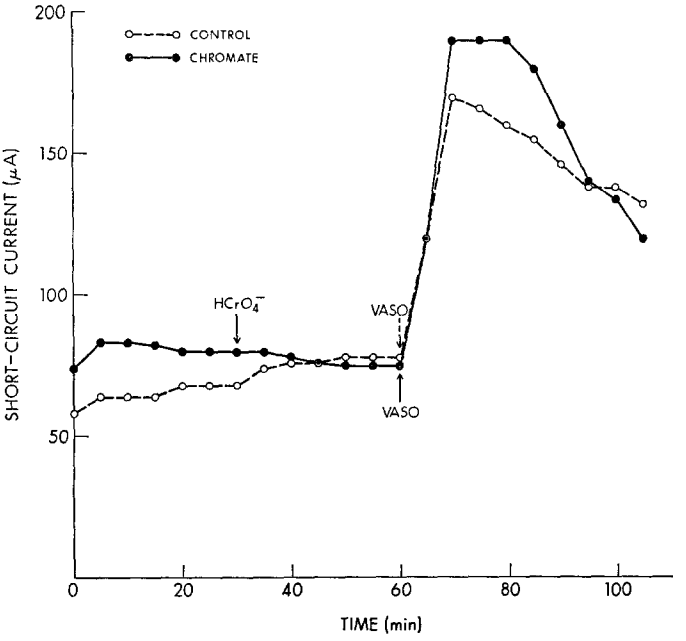

Fig. 4. Effect of 1 mM chromate on short-circuit current

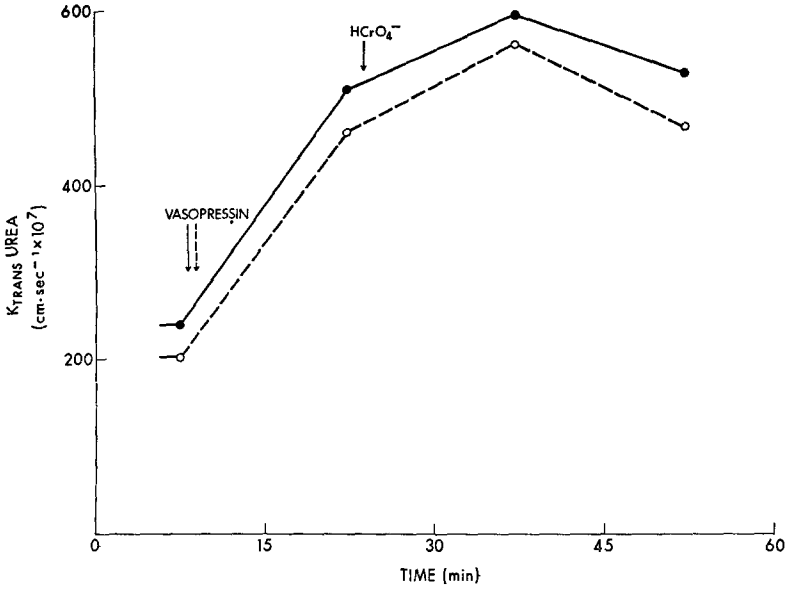

Fig. 5. Effect of addition of 1 mM chromate following vasopressin on K_{trans} urea. Test bladder shown with closed circles. Points are mean values for 3 paired experiments

luminal bathing medium of bladders already stimulated by vasopressin (Fig. 5). Chromate had no inhibitory effect on K_{trans} urea under these conditions, showing that it had to be added prior to vasopressin to inhibit urea movement.

Reversibility of Effect of Chromate

To determine whether the effect of chromate on the movement of urea was reversible, test bladders were exposed to 1 mM chromate in the luminal bathing medium for a total of 25 min (10 min preincubation, 15 min baseline period). Chromate was then removed, and the luminal and outside bathing mediums of both test and control bladders replaced 3 times with fresh phosphate-buffered Ringer's solutions. 1:10 phthalate-buffered Ringer's, pH 5.0, was then placed in the sacs, and osmotic water flow and K_{trans} urea redetermined. The results are shown in Fig. 6. Water flow continued at a rate equal to that of control after the removal of chromate; urea movement, however, remained significantly depressed, showing that the chromate effect on K_{trans} urea was irreversible. The length of time that the bladders were exposed to chromate was important; if the period of exposure was lengthened to 40 min, both water movement and K_{trans} urea were depressed (60% and 80%, respectively) following washout; if the period of exposure was decreased to 10 min, there was no inhibition of either urea or water movement following washout.

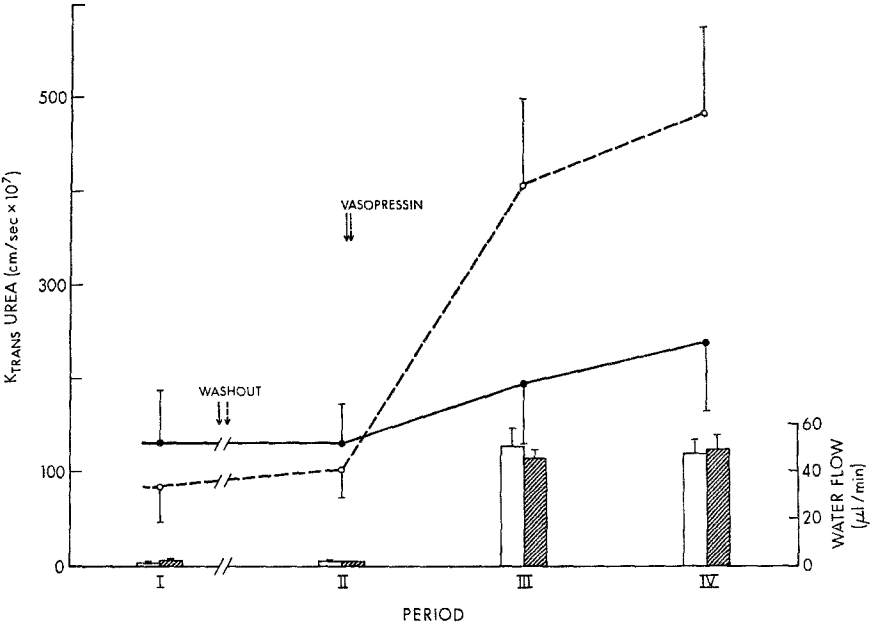

Fig. 6. Irreversibility of effect of chromate on K_{trans} urea. Test bladder (closed circles) received chromate at period I; washout of both test and control bladders following period I. Open bars represent water flow in control bladders; hatched bars in test bladders. Each period 15 min. Five paired experiments

Permeability of the Bladder to ^{51}Cr

To determine whether chromate crossed the bladder under the experimental conditions used, ^{51}Cr was added to the mucosal bathing medium containing 1 mM chromate both at pH 5.0 and 7.4. K_{trans} ^{51}Cr was then determined over the standard 45-min period used in our experiments. No counts could be detected in the serosal medium over this period of time; thus, the bladder appeared to be impermeable to chromate.

Discussion

10^{-4} M tannic acid and 10^{-3} M chromate, like phloretin, inhibit the movement of certain solutes across the toad bladder, but have no effect on vasopressin-stimulated osmotic water flow. While there is an overall similarity in the mode of action of these three agents, there are important differences as well. Tannic acid, like phloretin, markedly inhibits urea transport in the presence or absence of vasopressin, and irrespective of whether it is added before or after vasopressin. The inhibitory effect of tannic acid in the luminal solution on K_{trans} ^{14}C urea and ^{14}C ethylene glycol cannot be attributed to binding of the isotopes by the tannic acid polymer, since, when K_{trans} for these molecules was determined in the opposite direction (serosa to lumen), values comparable to those in Table 1 were obtained (Shuchter, Franki & Hays, *unpublished observations*). Like phloretin, tannic acid has no effect on the movement of ethanol or on resting short-circuit current or its initial rise following vasopressin. Short-circuit current following vasopressin persists at a higher level, however, an effect not seen with phloretin. We cannot account for this effect; it is possible that sodium entry across the luminal membrane of the vasopressin-treated bladder is accelerated by tannic acid.

There are two other respects in which the action of tannic acid differs from that of phloretin. The first is that at the lower concentration used (10^{-4} M), tannic acid reduces water movement across the bladder during the control period, prior to the addition of vasopressin. While the rates of water flow involved are very small, the effect appears to be a real one, and would suggest an effect of tannic acid on the few sites for water diffusion that exist in the absence of vasopressin. This inhibitory effect on baseline water movement was not seen in either of the two control periods with 5×10^{-4} M tannic acid. The second effect of tannic acid that distinguishes it from phloretin is its inhibitory action on K_{trans} ethylene glycol following vasopressin. This was seen with both concentrations of tannic acid, and the

inhibition was approximately 60% of the increment in K_{trans} produced by vasopressin. This suggests that the pathway for ethylene glycol is distinct from that of water in the vasopressin-treated bladder, a conclusion that did not emerge from our studies with phloretin, where both water and ethylene glycol were unaffected. This finding would also indicate that tannic acid and phloretin do not act at the same site in the membrane, and that tannic acid has a broader spectrum of action.

The studies of bladder permeability following removal of tannic acid and vasopressin (Table 2) again demonstrate the independence of water flow and solute movement. Water flow dropped sharply to a level close to baseline values, while K_{trans} urea rose, and K_{trans} ethylene glycol remained close to its vasopressin-stimulated level. The bladder behaved, then, as if the urea and ethylene glycol pathways were irreversibly "opened" to these solutes, while the pathway for water could return to its baseline state.[1]

The ability of tannic acid to impede the entry of ions and nonelectrolytes into the erythrocytes of a variety of species has been known for many years. Handovsky and Heubner demonstrated a reduction of nitrite entry in 1923; since then, inhibitory effects have been shown for the interchange of bicarbonate and chloride (Jacobs, Stewart & Butler, 1943), glycerol (Edelberg, 1952), ethylene glycol (Hunter, 1960), a number of sugars (Hunter, 1964), and urea (Hunter *et al.*, 1965). The probability that tannic acid was reacting with protein components of the membrane was suggested by Edelberg (1953), who showed that a monolayer of casein, a protein analogue of membrane proteins, solidified upon exposure to tannic acid, while monolayers of cholesterol, lecithin, cephalin, and oleic acid retained their liquid state. Further, the ionic permeability of lecithin spheres was not reduced by tannic acid, while the ohmic resistance of an albumin membrane was increased 25-fold by this agent. Comparable findings in protein films had been reported by Dean, Curtis and Cole (1940). Not all protein membranes were affected in this fashion, however; gelatin and casein membranes showed no change in resistance following tannic acid (Edelberg, 1953).

As far as the tannins themselves are concerned, they are a heterogeneous group of polyphenolic compounds of high molecular weight. In view of the complexity of the tannins, and the proteins with which they interact, it is not surprising that the precise nature of the cross-linking reaction is uncertain. It appears to involve multiple linkages, via hydrogen bonds to polypeptide groups, and via ionic bonds to basic protein groups such as lysine

1 It is possible that K_{trans} urea would have risen in the final period if tannic acid had remained in the bath, rather than being washed out. In either case, the independence of water and urea movement is demonstrated.

and arginine residues (Gustavson, 1966; Shuttleworth, Russell & Williams-Wynn, 1968). Quinone linkages may also be involved (Gustavson, 1966). In view of the complex and controversial nature of the interaction, it is not possible to go beyond the statement that the interaction of tannic acid with the luminal membrane of the toad bladder may have features in common with the cross-linking of proteins in other systems.

Turning to the effects of chromate on water and solute movement across the bladder, several findings deserve emphasis. First, 1 mM chromate has no effect in the absence of vasopressin. Second, the effect of chromate in vasopressin-treated bladders resembles that of phloretin: a significant depression of K_{trans} urea, and no effect on osmotic water flow, or on the movement of ethanol or ethylene glycol. Like phloretin, chromate has no effect on short-circuit current. We have not studied the effect of chromate on the movement of amides other than urea, so that it cannot yet be said that the spectrum of its action is identical to that of phloretin. Furthermore, there are two important differences between these agents: chromate must interact with the bladder prior to the addition of vasopressin to exhibit its inhibitory effect; second, the effect of chromate on K_{trans} urea appears to be irreversible, at least over the time period of the experiments shown in Fig. 6. The requirement that chromate be added prior to vasopressin is of particular interest, and suggests that the sites for the facilitated diffusion of urea which are stimulated by vasopressin can be blocked by chromate only in their "closed" configuration, prior to the addition of hormone. It is not clear from these experiments whether chromate is blocking new sites for urea transport, or is preventing vasopressin from accelerating urea transport across existing sites.

The nature of the interaction of chromate with the toad bladder is uncertain. It is probable that it takes place at the luminal membrane, in view of the impermeability of the bladder to ^{51}Cr placed in the luminal bathing medium. The chromate ion is predominantly in the $HCrO_4^-$ form at pH 5.0, and is in equilibrium with two other forms, as follows (Cotton & Wilkinson, 1972):

$$CrO_4^{2-} \rightleftharpoons HCrO_4^- \rightleftharpoons Cr_2O_7^{2-}. \quad (1)$$

The ratios of the three forms are 1:10:1, respectively. At pH 7.4, where the inhibitory effect on K_{trans} urea is less pronounced, and there is, in addition, a small inhibitory effect on water flow, CrO_4^{2-} is the predominant species, with a ratio of $CrO_4^{2-}/HCrO_4^-/Cr_2O_7^{2-}$ of 200:10:1. Thus, at pH 5.0, the $HCrO_4^-$ form of chromate is the most likely candidate for the effective inhibitor of urea movement. Since it is monovalent, it may not

be exerting its action by cross-linking a membrane protein; rather, it may be involved in uni-point binding to the membrane (Gustavson, 1962; Woodley, 1963).

We would conclude from these studies that tannic acid and chromate are capable of inhibiting the movement of urea across the toad bladder, with no inhibitory effect on active sodium transport or osmotic water flow. Our findings with chromate also suggest that the membrane component to which chromate binds is unavailable following vasopressin. Chromate appears to be an irreversible inhibitor of urea transport, a finding which may be of importance in future attempts to define the transport system for urea. Finally, we may tentatively characterize the urea transport system as involving a protein component of the cell membrane.

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