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Roles of Urinary Sodium Ion Concentration and pH in Promotion by Ascorbic Acid of Urinary Bladder Carcinogenesis in Rats[1]

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ABSTRACT

Since the sodium salt of ascorbic acid (AA) promoted two-stage urinary bladder carcinogenesis in rats, whereas AA itself did not, the roles of the urinary sodium ion concentration and pH on urinary bladder carcinogenesis were investigated. Male F344 rats were given 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in their drinking water for 4 weeks and then treated with basal diet containing 5% AA plus 3% sodium bicarbonate ($NaHCO_3$), 5% AA, 3% $NaHCO_3$ or 5% sodium L-ascorbate (SA), 5% SA plus 1% ammonium chloride (NH_4Cl) or 1% NH_4Cl, or no added chemical for 32 weeks. $NaHCO_3$ significantly increased the induction of neoplastic and preneoplastic lesions of the urinary bladder. Like SA, AA plus $NaHCO_3$ induced high incidences of neoplastic and preneoplastic lesions of the urinary bladder, whereas AA alone did not. NH_4Cl reduced the promoting activity of SA in urinary bladder carcinogenesis. These results suggest important roles for urinary sodium ion concentration and pH in modulating urinary bladder carcinogenesis. Moreover, AA was found to act as a copromoter under conditions of increased urinary pH and sodium ion concentration.

INTRODUCTION

The two-stage model of chemical carcinogenesis in the urinary bladder, consisting of initiation and promotion, has been demonstrated by several investigators (1–6). Recently, we examined the promoting activities of various chemicals in two-stage urinary bladder carcinogenesis in rats, using BBN,[3] a strong urinary bladder carcinogen, as initiator (7–12). Administration of 5% SA in the diet promoted urinary bladder carcinogenesis in rats (7), whereas administration of 5% AA in the diet did not (9). Urinary analysis showed that SA increased the urinary pH and sodium ion concentration, whereas AA did not (9). These results suggested that sodium ion has a key role in the promoting activity of AA and that elevation of the pH induced by sodium ingested as SA might be related to promotion of urinary bladder carcinogenesis.

In the present study, we examined the effects of AA plus $NaHCO_3$ and of SA plus NH_4Cl to determine the roles of urinary sodium ion concentration and pH on promotion by AA in two-stage urinary bladder carcinogenesis in rats.

MATERIALS AND METHODS

Animals. A total of 182 male 6-week-old F344 rats (Charles River Japan, Inc., Atsugi, Japan) were used. The rats were housed 5 per plastic cage with wood chips for bedding in an animal room with a 12-h light, 12-h dark cycle at 22 ± 2°C (SD) and 55 ± 10% relative humidity. Body weights, food consumption, and water intake were measured weekly up to week 14 and every other week from weeks 16 to 36. The amounts of food and water consumed in 2 consecutive days of a week were measured on a per cage basis.

Chemicals. BBN was from Tokyo Kasei Co., Tokyo, Japan. Food additive grade SA (Wako Pure Chemical Ind., Osaka, Japan), AA (Tanabe Seiyaku Co., Osaka, Japan), $NaHCO_3$ (Wako Pure Chemical Ind.), and NH_4Cl (Wako Pure Chemical Ind.) were used in experiments 1 and 2.

Experiment 1. Rats were randomly divided into 7 groups of 20 rats each. In the first 4 weeks they were given drinking water with 0.05% BBN, and then for 32 weeks they were given powdered basal diet (Oriental M; Oriental Yeast Co., Tokyo, Japan) containing 5% AA plus 3% $NaHCO_3$ (group 1), 5% AA (group 2), 3% $NaHCO_3$ (group 3), 5% SA (group 4), 5% SA plus 1% NH_4Cl (group 5), 1% NH_4Cl (group 6), or no added chemical (group 7, control group). The total observation period was 36 weeks.

For urine examination, fresh urine samples were obtained from 10 rats in each group in weeks 12, 24, and 36 by forced urination in the early morning. The pH was measured with a pH meter (Hitachi-Horiba pH meter, model F-7DE, Tokyo, Japan). In addition, 10 rats in each group were housed individually in metabolic cages without food or water for 4 h in the early morning for collections of urine samples. The osmolality was measured with an Osmette A instrument (Precision System, Inc., Natick, MA), and other parameters such as protein and occult blood were measured. Volumes of 2 ml of the remainder of samples were concentrated for microscopic examination of the urinary sediment.

In week 37, the rats were killed and their liver and kidneys were removed, weighed, and fixed in 10% phosphate-buffered formalin solution. The urinary bladder was inflated by intraluminal injection of 10% phosphate-buffered formalin solution and then cut into 8 strips for histological examination. For quantitative analysis, urinary bladder lesions were counted by light microscopy, the total length of the basement membrane was measured with a color video image processor (VIP-21CH; Olympus-Ikegami Tsushin Co., Tokyo, Japan), and the number of lesions per 10 cm of basement membrane was calculated. The liver and kidneys were also examined histologically.

Experiment 2. Rats were randomly divided into 7 groups of 6 rats each. The animals were given powdered diet (Oriental M) containing a test chemical as in experiment 1 but without BBN treatment for 4 weeks.

In week 4, for urinary electrolyte analysis, samples of the urine were obtained from 6 rats in each group. For collection of these samples, rats were housed individually in metabolic cages without food or water for 4 h in the early morning. Sodium, potassium, calcium, chlorine, phosphorus, magnesium, copper, and iron were analyzed in the Chunichi Clinic Center, Ohgaki, Japan. For measurement of the AA content of the urine, 4 rats in each group were placed in separated metabolic cages with glass collections tubes surrounded by ice and were given no food or water for 4 h in the early morning of different days in week 4. The urine was stored in a freezer. The following day, the contents of total AA and DA in the urine were measured by the 2,4-dinitrophenylhydrazine calorimetric method at the Japan Food Research Laboratories, Tokyo, Japan.

RESULTS

Experiment 1. Rats in test groups showed no toxic symptoms due to the chemicals, but several rats given AA plus $NaHCO_3$ (group 1) or SA (group 4) had hematuria in later stages of the experiment. Data on the weights of the body and urinary

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[3] The abbreviations used are: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; AA, ascorbic acid; SA, sodium L-ascorbate; DA, dehydroascorbic acid.

Table 1 *Average body and urinary bladder weights and food and water consumptions (weeks 5–36) of rats in experiment 1*

Group	Test chemicals	No. of rats	Body wt (g)		Urinary bladder wt		Av. food consumption (g/rat/day)	Av. water consumption (g/rat/day)
			Initial	Final	Absolute (g)	Relative (organ/body wt %)		
1	AA + $NaHCO_3$	20	132.6 ± 5.2[a]	394.0 ± 17.6[c, e, f]	0.85 ± 0.92[b, d, f]	0.22 ± 0.25[b, d, f]	16.0	32.3
2	AA	20	133.4 ± 4.6	424.4 ± 18.4[g]	0.16 ± 0.03	0.04 ± 0.01	18.0	22.0
3	$NaHCO_3$	20	132.8 ± 5.6	413.9 ± 25.1[i]	0.26 ± 0.15[h]	0.06 ± 0.04[h]	15.3	24.7
4	SA	20	132.1 ± 6.6	405.6 ± 28.8[k]	0.51 ± 0.69[j]	0.13 ± 0.15[j]	14.7	26.7
5	SA + NH_4Cl	20	133.8 ± 5.5	395.0 ± 18.3[l]	0.20 ± 0.06	0.05 ± 0.02	18.7	26.7
6	NH_4Cl	20	133.5 ± 6.7	416.7 ± 20.7[m]	0.15 ± 0.02	0.04 ± 0.01	18.3	21.3
7		20	133.6 ± 4.4	438.1 ± 18.4	0.17 ± 0.04	0.04 ± 0.01	15.0	19.7

[a] Mean ± SD.

Values for the following groups were significantly different: groups 1 and 2, [b] $P < 0.01$, [c] $P < 0.001$; groups 1 and 3, [d] $P < 0.05$, [e] $P < 0.01$; groups 1 and 7, [f] $P < 0.01$; groups 2 and 7, [g] $P < 0.05$; groups 3 and 7, [h] $P < 0.05$, [i] $P < 0.01$; groups 4 and 7, [j] $P < 0.05$, [k] $P < 0.01$; groups 5 and 7, [l] $P < 0.01$; groups 6 and 7, [m] $P < 0.01$.

Table 2 *Induction of urinary bladder lesions in rats treated with BBN followed by test chemicals*

Group	Test chemicals	Effective no. of rats	Incidence of simple hyperplasia (%)	Incidence of papillary or nodular hyperplasia (%)	Papilloma		Carcinoma	
					Incidence (%)	No./10 cm of BM[a]	Incidence (%)	No./10 cm of BM
1	AA + $NaHCO_3$	20	18 (90)[b, f]	20 (100)[b, f]	18 (90)[b, e]	2.27 ± 1.35[c, d, g, q]	19 (95)[c, g]	2.76 ± 1.44[c, d, g]
2	AA	20	9 (45)	12 (60)	8 (40)	0.46 ± 0.70	4 (20)	0.18 ± 0.37
3	$NaHCO_3$	20	19 (95)[i]	20 (100)[j]	15 (75)	0.94 ± 0.82	16 (80)[i]	1.03 ± 0.70[h]
4	SA	20	18 (90)[m]	20 (100)[n]	19 (95)[k, m]	1.75 ± 1.02[k, m]	17 (85)[k, n]	1.93 ± 1.18[k, n]
5	SA + NH_4Cl	20	19 (90)[o]	19 (95)[p]	13 (65)	0.93 ± 0.97	9 (45)	0.69 ± 1.02
6	NH_4Cl	20	11 (55)	4 (20)	5 (25)	0.37 ± 0.66	4 (20)	0.20 ± 0.40
7		20	10 (50)	6 (30)	11 (55)	0.81 ± 0.94	5 (25)	0.42 ± 0.79

[a] BM, basement membrane.

Values for the following groups were significantly different: groups 1 and 2, [b] $P < 0.01$, [c] $P < 0.001$; groups 1 and 3, [d] $P < 0.001$; groups 1 and 7, [e] $P < 0.05$, [f] $P < 0.01$, [g] $P < 0.001$; groups 3 and 7, [h] $P < 0.05$, [i] $P < 0.01$; [j] $P < 0.001$; groups 4 and 5, [k] $P < 0.05$, [l] $P < 0.01$; groups 4 and 7, [m] $P < 0.01$, [n] $P < 0.001$; groups 5 and 7, [o] $P < 0.01$, [p] $P < 0.001$.

[q] Mean ± SD.

bladder and consumptions of food and water are summarized in Table 1. The final average body weights of rats in groups 1 to 6 given test chemicals were significantly lower than that of control group 7. The final average body weight of group 1 given AA plus $NaHCO_3$ was less than that of group 2 given AA alone. The absolute and/or relative urinary bladder weights in groups 1, 3, and 4 were significantly higher than those in control group 7. Moreover, there were significant differences in the urinary bladder weights of groups 1 and 2 and of groups 1 and 3. No reduction in the average food intake was observed in any test group. Water consumptions were higher in groups 1, 3, 4, and 5 than in the control, the value in group 1 being particularly high. Rats in groups 1, 3, and 4 had more tumors of the urinary bladder than rats in groups 2, 5, 6, and 7. The number of multiple tumors was highest in groups 1 and 4 followed by group 3.

Histological findings on the urinary bladder epithelium of rats are summarized in Table 2. As described previously (13), the epithelial lesions of the urinary bladder were classified into 4 types: simple hyperplasia; papillary or nodular hyperplasia; papilloma; and carcinoma. The incidences of papillary or nodular hyperplasia in groups 1, 3, 4, and 5 were significantly higher than that in group 7. The incidences of papilloma in groups 1 and 4 were significantly higher than that in group 7 and the incidences of carcinoma were significantly higher in groups 1, 3, and 4 than that in group 7. Moreover, the incidences of papilloma and carcinoma in groups 1 and 4 were significantly different from those in groups 2 and 5, respectively. The numbers of papillomas and/or carcinomas were significantly higher in groups 1, 3, and 4 than in group 7. Treatment with AA plus $NaHCO_3$ (group 1) resulted in much higher inductions of papillomas and carcinomas than treatment with $NaHCO_3$ only (group 3). Treatment with SA plus NH_4Cl (group 5) resulted in significantly fewer papillomas and carcinomas than treatment with SA alone (group 4), the numbers of papillomas and carcinomas in group 5 being similar to those in control group 7. Treatments with AA (group 2) and NH_4Cl (group 6) also tended to decrease the inductions of neoplastic lesions by BBN (group 7).

Table 3 *Urine analysis of rats treated with BBN followed by test chemicals (data in week 36)*

Group	Test chemicals	No. of rats examined	pH	Osmolality (mOsmol/kg H_2O)	Crystals
1	AA + $NaHCO_3$	10	7.25 ± 0.32[a, b]	1130 ± 204[b]	++
2	AA	10	6.12 ± 0.11[b]	2102 ± 349	±
3	$NaHCO_3$	10	7.89 ± 0.51[b]	1743 ± 325[c]	+ ~ ++
4	SA	10	7.48 ± 0.23[b]	1425 ± 344[b]	++
5	SA + NH_4Cl	10	6.49 ± 0.30	2041 ± 264	+ ~ ++
6	NH_4Cl	10	5.78 ± 0.07[b]	2254 ± 126	±
7		10	6.69 ± 0.35	2099 ± 379	±

[a] Mean ± SD.

[b] Significantly different from group 7 at $P < 0.01$.

[c] Significantly different from group 7 at $P < 0.05$.

Results of urine analysis in experimental week 36 are shown in Table 3. The urinary pH was increased in groups 1, 3, and 4 but significantly decreased in groups 2 and 6. The urinary pH in group 5 was almost the same as that in group 7. The osmolality was low in groups 1, 3, and 4. Increase of crystals of $MgNH_4PO_4$ was observed in the urinary sediment in groups 1, 3, 4, and 5. Urine analyses in weeks 12 and 24 gave results similar to those in week 36.

Experiment 2. The sodium ion concentrations of the urine of rats in groups 1, 3, 4, and 5 were higher than that of group 7 (Table 4). However, that of group 6 was lower than that of group 7. The copper ion concentrations of the urine of rats in groups 1, 4, and 5 were lower than that of group 7, whereas those of groups 2 and 3 were significantly higher than that of group 7. Significant increases or decreases in the levels of some other electrolytes from those in group 7 were observed in the

Table 4 *Urinary electrolytes of rats treated with test chemicals for 4 weeks*

Group	Test chemicals	Na (meq/liter)	K (meq/liter)	Ca (meq/liter)	Cl (mg/dl)	P (mg/dl)	Mg (mg/dl)	Cu (mg/dl)	Fe (mg/dl)
1	AA + $NaHCO_3$	471 ± 116[a, b]	168.7 ± 43.7	9.2 ± 4.7	109 ± 47	80.1 ± 46.0	32.6 ± 26.3	57 ± 5[c]	80 ± 15
2	AA	127 ± 22	237.3 ± 60.0	11.0 ± 1.2	153 ± 45	149.0 ± 115.0	66.7 ± 13.8	80 ± 10[c]	91 ± 27
3	$NaHCO_3$	420 ± 56[b]	175.5 ± 38.3	15.3 ± 5.0[c]	142 ± 16	16.6 ± 10.4[c]	38.1 ± 17.5	77 ± 4[c]	55 ± 7
4	SA	316 ± 90[b]	144.0 ± 39.1	9.3 ± 6.0	103 ± 45	98.1 ± 61.0	24.8 ± 15.3[c]	51 ± 3[b]	63 ± 16
5	SA + NH_4Cl	340 ± 57[b]	154.9 ± 43.3	7.1 ± 2.8	231 ± 50[c]	48.0 ± 46.6	37.0 ± 23.7	54 ± 6[b]	58 ± 13
6	NH_4Cl	73 ± 27[c]	183.8 ± 62.1	6.1 ± 3.5	272 ± 103[c]	63.7 ± 45.6	32.5 ± 17.3	68 ± 10	69 ± 20
7		121 ± 36	185.1 ± 24.6	7.8 ± 3.3	156 ± 37	49.0 ± 22.6	49.5 ± 17.9	68 ± 7	61 ± 20

[a] Mean ± SD.
[b] Significantly different from group 7 at $P < 0.01$.
[c] Significantly different from group 7 at $P < 0.05$.

Table 5 *Total ascorbic acid and dehydroascorbic acid in the urine of rats treated with test chemicals for 4 weeks*

Group	Test chemicals	No. of rats examined	Total ascorbic acid (mg/100 ml)	Dehydroascorbic acid (mg/100 ml)
1	AA + $NaHCO_3$	4	45.7 ± 5.8[a, b]	3.7 ± 0.3[b]
2	AA	4	96.4 ± 19.3[b]	5.9 ± 0.9[b]
3	$NaHCO_3$	4	0.1 ± 0.2[c]	0.1 ± 0.2[c]
4	SA	4	66.6 ± 10.7[b]	4.7 ± 0.8[b]
5	SA + NH_4HCl	4	39.9 ± 12.8[d]	4.8 ± 0.9[b]
6	NH_4Cl	4	0.9 ± 0.1[d]	0.9 ± 0.1[d]
7		4	0.5 ± 0.1	0.5 ± 0.1

[a] Mean ± SD.
[b] Significantly different from group 7 at $P < 0.001$.
[c] Significantly different from group 7 at $P < 0.05$.
[d] Significantly different from group 7 at $P < 0.01$.

Table 6 *Summary of relationship between changes in urinary parameters and promoting potential*

Test chemicals	Urine pH	Urine Na^+	Urine Total ascorbic acid	Promoting potential
AA + $NaHCO_3$	↑[a]	↑	↑	+++
AA	↓	→	↑	−
$NaHCO_3$	↑	↑	→	+
SA	↑	↑	↑	++ ~ +++
SA + NH_4Cl	→	↑	↑	±
NH_4Cl	↓	↓	→	−

[a] ↑, increase; ↓, decrease; →, no change; +++, marked; ++, moderate; +, slight; ±, very slight; −, no change.

test groups. The urinary contents of total AA and DA in rats treated with test chemicals are shown in Table 5. The values of total AA and DA were significantly higher in groups 1, 2, 4, 5, and 6 than in group 7, while the values in group 3 were significantly lower than those in group 7. However, it is considered that the values in groups 3 and 6 were similar to those of controls and were thus different from those of groups 1, 2, 4, and 5.

DISCUSSION

In the present study, we confirmed that SA promotes urinary bladder carcinogenesis, whereas AA does not. $NaHCO_3$ also exhibited promoting activity in urinary bladder carcinogenesis. The most interesting finding in the present study was that AA plus $NaHCO_3$ showed potent promoting activity in urinary bladder carcinogenesis like that of SA, whereas NH_4Cl inhibited the promoting activity of SA.

Data on the relationship between promoting potential and changes in urinary parameters are summarized in Table 6. $NaHCO_3$ itself showed promoting activity in urinary bladder carcinogenesis in the present study. Sodium *o*-phenylphenate (8) and sodium erythorbate (9) promoted urinary bladder carcinogenesis in rats, whereas *o*-phenylphenol and erythorbic acid did not. Sodium saccharin has promoting activity but saccharin acid does not seem to have this activity (1–3). Sodium *o*-phenylphenate, sodium erythorbate, and sodium saccharin increased the urinary pH and sodium ion concentration of the urine. Therefore, increases of the sodium ion concentration and pH of the urine are apparently important factors for urinary bladder carcinogenesis.

Urinary analyses showed differences in the contents of total AA and DA of groups treated with AA plus $NaHCO_3$ and $NaHCO_3$, but not in other parameters, such as the pH, osmolality, and electrolytes. It is considered that the values of the contents of total AA and DA in rats given $NaHCO_3$ were similar to those of controls, although significantly different. The promoting activity of AA plus $NaHCO_3$ on urinary bladder carcinogenesis was greater than that of $NaHCO_3$. Therefore, AA acts as a copromoter under conditions of increased urinary pH and sodium ion concentration in urinary bladder carcinogenesis and the promoting activity of SA is nonspecific. A high intracellular concentration of AA or its metabolites might be induced in the observed changes in composition of the urine. Further investigations are needed on this point.

The membrane potential of the epithelium in the early stage of urinary bladder carcinogenesis is significantly increased by BBN or sodium saccharin treatment (14). Since the apical membrane potential of the cell depends largely on the permeability of the sodium ion, it reflects the activity of the sodium ion channel, which is essential in sodium ion transport across the urinary bladder epithelium. Therefore, it seems likely that a high concentration of sodium ion in the urine produces high levels of intracellular sodium ion in the urinary bladder epithelium and these may induce elevation of the intracellular pH. In general, a high intracellular concentration of sodium ion is thought to be related to cellular proliferation (15, 16). In addition, there is a good correlation between increase in the intracellular pH and DNA synthesis in cells (17). Increases in the sodium ion content of the urine result in proliferation of the urinary bladder epithelial cells (18, 19) and renal pelvic epithelial cells (20). This phenomenon may be correlated with the promoting activity of sodium ion in urinary bladder carcinogenesis.

In the present study, treatment with NH_4Cl reduced the promoting activity of SA on urinary bladder carcinogenesis and did not cause an increase in the urinary pH, although SA increased the sodium ion concentration of the urine. Moreover, previously we found that sodium hippurate did not show promoting activity in urinary bladder carcinogenesis in rats initiated with BBN (21), although it increases the sodium content of the urine but did not increase the urinary pH (21). Furthermore, acetazolamide did not show promoting activity in BBN urinary bladder carcinogenesis of rats; it elevated the urinary pH but did not increase to the sodium ion concentration of the urine (21). Thus, increases of both sodium ion concentration and pH of the urine are important for urinary bladder carcino-

genesis. Recently it was reported that proliferative alteration of the bladder epithelium induced by sodium saccharin is correlated with changes in urinary sodium ion and urinary pH (22). Calcium ion is also known to be important in the proliferation of bladder epithelium cells (23). However, in the present study no increase in the calcium ion content of the urine was observed in any test group. Treatment with AA plus $NaHCO_3$, $NaHCO_3$, or SA induced low urinary osmolality as the results of polyuria. However, this change probably did not influence promoting activity in urinary bladder carcinogenesis, because acetazolamide and *o*-phenylphenol also induced very low osmolality of the urine.

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