

Synergism by Sodium L-Ascorbate but Inhibition by L-Ascorbic Acid for Sodium Saccharin Promotion of Rat Two-Stage Bladder Carcinogenesis¹

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ABSTRACT

Since both sodium L-ascorbate (Na-AsA) and sodium saccharin (Na-Sac) promote two-stage bladder carcinogenesis in rats, synergism of the two chemicals was investigated with special reference to the role of urinary pH and Na⁺ concentration. Male F344 rats were given 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in the drinking water for 4 wk and then treated with basal diet containing 5% Na-Sac, 5% Na-AsA, 5% Na-Sac plus 5% Na-AsA, 5% L-ascorbic acid (AsA), 5% Na-Sac plus 5% AsA, or no added chemical for 32 wk. Treatment with Na-Sac or Na-AsA alone significantly increased the induction of neoplastic and preneoplastic lesions of the bladder. Na-Sac plus Na-AsA also induced these bladder lesions significantly when compared with the controls, and the number of lesions was greater than the sum of the lesions in the groups treated with Na-Sac alone or Na-AsA alone. In contrast, the induction of carcinomas and papillomas in rats treated with Na-Sac plus AsA was not significantly different from the controls. In addition Na-Sac plus Na-AsA produced an elevation of urinary pH and Na⁺ concentrations, although the increases were not different from those in rats fed Na-Sac or Na-AsA alone. Na-Sac plus AsA, however, did not cause elevation of urinary pH, although it increased urinary Na⁺ concentration. Thus, the bladder carcinogenesis promotion by Na-Sac was synergized by Na-AsA and inhibited by AsA. This modulation was associated with changes of urinary pH and Na⁺ concentration.

INTRODUCTION

It is generally accepted that environmental factors may influence the development of cancer in humans. Epidemiological data, for example, demonstrated the importance of chemicals in occupational bladder cancer (1). However, there is no convincing evidence for associations with nonoccupational bladder cancer in humans. Humans are exposed to numerous environmental chemicals in their life span, and the chemicals may act in combination to produce cancer. In fact, synergistic and additive tumor induction by multiple carcinogens has been demonstrated in previous experiments (2-4), and our laboratory reported synergistic and summation effects in the induction of bladder cancer in rats (5, 6).

The concept of two-step chemical carcinogenesis in the bladder of rats is now well accepted. Many bladder cancer promoters have been found. Na-Sac³ was the first found to exert promoting activity in bladder carcinogenesis in rats (7-9). We recently demonstrated that increased urinary pH and Na⁺ concentration resulting from the feeding of large amounts of sodium salts play a key role in the promotion of bladder carcinogenesis in rats (10-13), and this is consistent with other reports (14, 15). For instance, sodium bicarbonate (NaHCO₃) significantly increased the induction of neoplastic lesions of the bladder (10, 12), and AsA amplified this promotion by NaHCO₃, whereas

AsA alone did not promote bladder carcinogenesis. In addition, ammonium chloride, which acidifies the urine, reduced the promoting activity of Na-AsA in bladder carcinogenesis (12).

In order to further elucidate the mechanism of the promotion by sodium salts in bladder carcinogenesis and to evaluate possible synergism of promoters of sodium salts, we performed the following study in rats, with special reference to effects on urinary pH and Na⁺ concentration.

MATERIALS AND METHODS

Animals. A total of ninety-three 6-wk-old male 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 60 ± 10% relative humidity. Body weights were measured every 4 wk during the experiment. Food consumption and water intake were measured at wk 12 and 24. The amounts of food and water consumed in 2 consecutive days of a week were measured on a per cage basis.

Chemicals. BBN was obtained from Tokyo Kasei Co., Tokyo, Japan. Food-additive grades Na-Sac, Na-AsA, and AsA (all purchased from Wako Pure Chemical Industries, Osaka, Japan) were used in this experiment.

Experimental Design. Rats were randomly divided into 6 groups of 15 or 16 rats each. In the first 4 wk they were given drinking water with 0.05% BBN, and then for 32 wk they were given powdered basal diet (Oriental MF; Oriental Yeast Co., Tokyo, Japan) as a control (Group 1) or fed the diet containing 5% Na-Sac (Group 2), 5% Na-AsA (Group 3), 5% Na-Sac plus 5% Na-AsA (Group 4), 5% AsA (Group 5), and 5% Na-Sac plus 5% AsA (Group 6). The total observation period was 36 wk.

For urine examination, fresh urine samples were obtained from five rats in each group during wk 14, 24, and 34 by forced urination in the morning (9:00-10:00 a.m.). pH was measured with a pH meter (Model F-7DE pH meter; Hitachi-Horiba, Tokyo, Japan). For urinary electrolyte analysis, samples of urine were obtained from five rats in each group during wk 15, 25, and 35, housed individually in metal metabolic cages without food or water for 4 h in the morning (8:00-12:00 a.m.). The urine samples thus collected were analyzed for sodium, potassium, calcium, chloride, phosphorus, and magnesium. Analytical methods were flame photometry (Flame-30C photometer; Jasco Medical Industries, Tokyo, Japan) for sodium and potassium, the *o*-cresolphthalein complexone method (Model-ACA-8000 chemical analyzer; Olympus Optical Co., Ltd., Tokyo, Japan) for calcium, a chloride meter (Model CL-12; Jasco Medical Industries) for chloride, modification of the phosphomolybdate method (Model ACA-8000 chemical analyzer) for phosphorus, and reaction with Calmagite (Model ACA-8000 chemical analyzer) for magnesium. The osmolality was measured with an Osmette A instrument (Precision System, Inc., Natick, MA), and occult blood was measured during wk 15, 25, and 35. Two ml of the samples were concentrated for microscopic examination of the urinary sediment.

At the beginning of wk 37, the rats were sacrificed under ether anesthesia, and their livers and kidneys were removed and weighed. Urinary bladders were inflated by intraluminal injection of 10% phosphate-buffered formalin solution, and after fixation, they were divided sagittally and weighed, and each half was cut into four strips for histological examination. For quantitative analysis, urinary bladder lesions were counted by light microscopy, the total length of the basement membrane being measured with a color video image processor (VIP-21CH; Olympus-Ikegami Tsushin Co., Tokyo, Japan), and the

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³ The abbreviations used are: Na-Sac, sodium saccharin; Na-AsA, sodium L-ascorbate; AsA, L-ascorbic acid; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; PN, papillary or nodular.

Table 1 Average body weight, food consumption, and water consumption data

Group	Chemicals	Effective no. of rats	Final body wt (g)	Food consumption (24 wk) (g/rat/day)	Water consumption (24 wk) (ml/rat/day)
1	No treatment	15	411 ± 21 ^a	12.2	18.6
2	Na-Sac	15	359 ± 18 ^{b,c}	12.1	23.3
3	Na-AsA	16	357 ± 25 ^{b,c}	11.2	21.3
4	Na-Sac + Na-AsA	13	324 ± 18 ^b	11.2	23.8
2	Na-Sac	15	359 ± 18 ^{b,d}	12.1	23.3
5	AsA	16	371 ± 17 ^{b,d}	10.7	18.6
6	Na-Sac + AsA	16	336 ± 23 ^b	11.7	22.2

^a Mean ± SD.
^b P < 0.01 (significantly different from Group 1, Student's *t* test).
^c P < 0.01 (significantly different from Group 4, Student's *t* test).
^d P < 0.01 (significantly different from Group 6, Student's *t* test).

Table 2 Induction of preneoplastic and neoplastic lesions in the bladder

Group	Chemicals	Effective no. of rats	Papillary or nodular hyperplasia		Papilloma		Carcinoma	
			Incidence	No./10 cm of BM ^a	Incidence	No./10 cm of BM	Incidence	No./10 cm of BM
1	No treatment	15	4 (26.7) ^b	0.4 ± 0.6 ^c	4 (26.7)	0.5 ± 0.9	0 (0)	0
2	Na-Sac	15	14 (93.3) (a) ^d	4.0 ± 2.4 (a)(b) ^e	9 (60.0) (c) ^f	0.7 ± 0.7 (b)	5 (33.3) (b)	0.6 ± 1.0 (b)(d) ^d
3	Na-AsA	16	15 (93.8) (a)	7.1 ± 4.3 (a)(c)	13 (81.3) (a)	1.5 ± 1.2 (b)(d)	11 (68.8) (a)(c)	1.7 ± 1.4 (a)(b)
4	Na-Sac + Na-AsA	13	13 (100) (a)	16.0 ± 11.0 (a)	13 (100) (a)	5.2 ± 3.3 (a)	13 (100) (a)	3.7 ± 2.0 (a)
2	Na-Sac	15	14 (93.3) (a)	4.0 ± 2.4 (a)(e) ^f	9 (60.0)	0.7 ± 0.7	5 (33.3)	0.6 ± 1.0
5	AsA	16	4 (25.0)	0.2 ± 0.4 (f) ^f	5 (31.3)	0.4 ± 0.7	2 (12.5)	0.1 ± 0.3
6	Na-Sac + AsA	16	10 (62.5)	0.9 ± 0.8	4 (25.0)	0.3 ± 0.5	1 (6.3)	0.1 ± 0.2

^a BM, basement membrane.
^b Numbers in parentheses, percentage.
^c Mean ± SD.
^d Significantly different from Group 1, two-tailed Fisher's exact probability test or Student's *t* test: (a), P < 0.01; (d), P < 0.05.
^e Significantly different from Group 4, two-tailed Fisher's exact probability test or Student's *t* test: (b), P < 0.01; (c), P < 0.05.
^f Significantly different from Group 6, two-tailed Fisher's exact probability test or Student's *t* test: (e), P < 0.01; (f), P < 0.05.

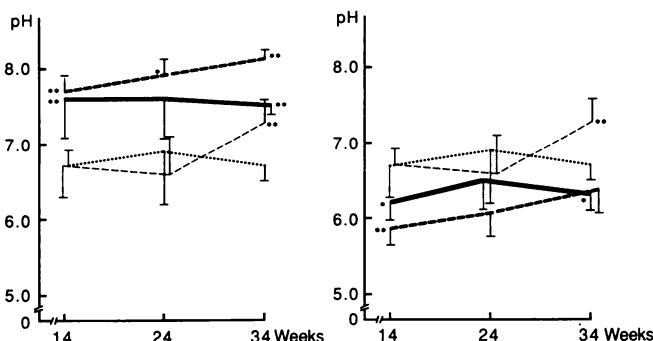


Fig. 1. Average urinary pH of rats treated with no chemical (....., Group 1), Na-Sac (----, Group 2), Na-AsA (----, Group 3), or Na-Sac plus Na-AsA (—, Group 4) (left) and no chemical (....., Group 1), Na-Sac (----, Group 2), AsA (----, Group 5), or Na-Sac plus AsA (—, Group 6) (right). *, P < 0.05; **, P < 0.01 (significantly different from Group 1, Student's *t* test). The data are for the mean ± SD.

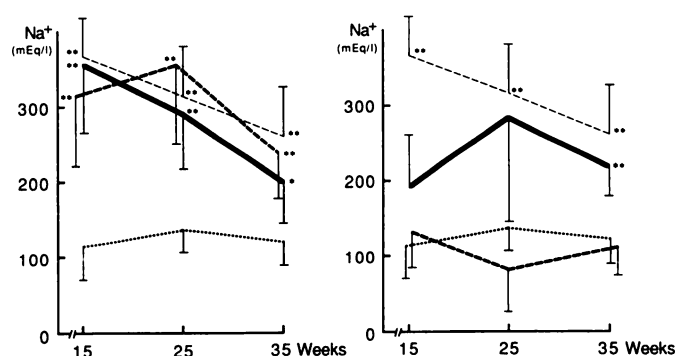


Fig. 2. Average urinary Na⁺ concentration of rats treated with no chemical (....., Group 1), Na-Sac (----, Group 2), Na-AsA (----, Group 3), or Na-Sac plus Na-AsA (—, Group 4) (left) and no chemical (....., Group 1), Na-Sac (----, Group 2), AsA (----, Group 5), or Na-Sac plus AsA (—, Group 6) (right). *, P < 0.05; **, P < 0.01 (significantly different from Group 1, Student's *t* test). The data are for the mean ± SD.

numbers of lesions per 10 cm of basement membrane were calculated (9).

Statistical Analysis. Data concerning incidences of lesions were analyzed for statistical significance with Fisher's exact probability test. Other data were analyzed using Student's *t* test.

RESULTS

Data on final body weights and average food and water consumptions at wk 24 are shown in Table 1. The final body weights of rats in Groups 2 to 6 given test chemicals were significantly lower than the controls (Group 1). In particular, body weights in Groups 4 and 6 were significantly decreased compared with Groups 2 and 3 or Groups 2 and 5, respectively. Average food consumption in Groups 2 to 6, except Group 5

given AsA, was almost the same as for Group 1. Increases of average water consumption were obtained in Groups 2 to 6, except Group 5. Rats in Group 5 showed slight decreases in food and water consumptions; however, their body weights were higher than those of the other groups (Groups 2, 3, 4, and 6) given chemicals. The rats in Group 4 were found dead at wk 32 and 33; the cause of death could not be established. Although these rats had bladder tumors, they were not included in the effective numbers, because of autolysis.

Macroscopically, bladders of rats in Groups 2, 3, and, particularly, Group 4 had multiple, large tumors. No stone formation was observed in any of the groups.

Histological findings concerning the bladder epithelium are summarized in Table 2. Epithelial lesions found in the bladder

Table 3 Urine analysis data of rats at wk 35

Group	Chemicals	No. of rats	Electrolytes		
			Potassium (mEq/liter)	Calcium (mEq/liter)	Chlorine (mEq/liter)
1	No treatment	5	213.8 ± 39.0 ^a	4.5 ± 2.0	145.8 ± 35.7
2	Na-Sac	5	196.5 ± 18.4 (a) ^b	5.4 ± 1.9	101.6 ± 33.3 (b) ^b
3	Na-AsA	4	106.8 ± 31.6 (c) ^c	10.9 ± 0.5 (d) ^c	64.5 ± 19.7 (c)
4	Na-Sac + Na-AsA	5	71.1 ± 16.6 (c)	12.8 ± 6.4	45.4 ± 17.5 (c)
2	Na-Sac	5	196.5 ± 18.4 (e) ^d	5.4 ± 1.9 (f) ^d	101.6 ± 33.3
5	AsA	4	230.8 ± 77.2 (b)	11.5 ± 9.2	134.0 ± 58.5 (b)
6	Na-Sac + AsA	5	123.5 ± 22.3 (a)(c)	9.9 ± 2.9 (d)	110.0 ± 28.2 (a)

^a Mean ± SD.

^b Significantly different from Group 4, Student's *t* test: (a), *P* < 0.01; (b), *P* < 0.05.

^c Significantly different from Group 1, Student's *t* test: (c), *P* < 0.01; (d), *P* < 0.05.

^d Significantly different from Group 6, Student's *t* test: (e), *P* < 0.01; (f), *P* < 0.05.

Table 4 Summary of experimental results

Chemicals	Promoting potential	Urine pH	Urine Na ⁺
Na-Sac	+	↑	↑
Na-AsA	+	↑	↑
Na-Sac + Na-AsA	+++	↑	↑
AsA	-	↓	→
Na-Sac + AsA	-	↓	↑

were classified into four types: simple hyperplasia; PN hyperplasia, a putative preneoplastic lesion; papilloma; and carcinoma as described previously (16, 17). Incidences and numbers per 10 cm of basement membrane of carcinoma were significantly higher in Groups 2, 3, and 4 than in Group 1. Also, the number of carcinomas in Group 4 was significantly higher than in Groups 2 and 3. Incidences and numbers of papillomas were significantly higher in Groups 3 and 4 than in Group 1, and the numbers in Group 4 were greater than the sum of those in Groups 2 and 3, as with carcinomas. Inductions of PN hyperplasia in rats of Groups 2 to 4 show the same results as for carcinomas. In contrast, incidences and numbers of carcinomas and papillomas in Groups 5 and 6 were not significantly different from Group 1 nor for Group 6 compared with Group 2. The numbers of PN hyperplasia were significantly lower in Group 6 than in Group 2 and in Group 5 than in Group 6.

Figs. 1 and 2 show the results of urinary pH and Na⁺ concentration of rats in Groups 1 to 6. The urinary pH was increased in Groups 3 and 4 at wk 14, 24, and 34 and Group 2 at wk 34. Values of Group 3 were always higher than those of Group 2. Groups 5 and 6 showed a decreasing tendency compared with Group 1 during the experiment. Groups 2, 3, and 4 showed significant elevation of urinary Na⁺ concentration, but among the 3 groups (Groups 2 to 4) treated with Na-Sac, Na-AsA, and Na-Sac plus Na-AsA, Group 4 showed a tendency for less of an increase than did Groups 2 or 3. Group 6 induced an increased urinary Na⁺ concentration compared with Group 1 and with Group 2, whereas Group 5 did not. Other urinary parameters at wk 35 are shown in Table 3. K⁺ concentrations in Groups 3, 4, and 6 (given Na-AsA) were significantly lower than in Group 1. The Cl⁻ concentration showed decreased values in Groups 1 to 6, except Group 5. The concentrations of K⁺ and Cl⁻ in Group 4 were significantly lower than in Groups 5 and 6. These results were similar to those at wk 15 and 25.

Values of urinary osmolality were lower in Groups 3, 4, and 6 than in Group 1 at wk 15 and 25. Hematuria was seen in rats of Group 4. In the urinary sediment, MgNH₄PO₄ crystals, based on their morphological appearance by light microscopy, were detected more in Groups 2 and 6 at all time points examined.

DISCUSSION

In the present study, we detected a synergistic effect of 2 rat bladder cancer promoters, Na-Sac and Na-AsA, and an anti-promoting effect of AsA on Na-Sac bladder carcinogenesis promotion. We also confirmed that Na-Sac and Na-AsA promoted bladder carcinogenesis, whereas AsA did not.

Data on the relation between promoting potential and changes in urinary parameters are summarized in Table 4. This study confirmed that increases of urinary pH and Na⁺ concentration are apparently important factors for bladder carcinogenesis promotion by Na-AsA or Na-Sac, as indicated previously (10–13). AsA showed the same findings which we reported previously (10–12) concerning urinary parameters, such as pH and Na⁺ concentration, and its nonpromoting potential. In addition, the promoting potential of simultaneous treatment with Na-Sac and Na-AsA correlated with the elevation of urinary pH and increased Na⁺ concentration. However, there was no correlation between the magnitude of the increases of urinary pH and Na⁺ concentration and the extent of their promoting activities. In addition, the combined treatment with Na-Sac and AsA did not exert promoting activity.

In the present study, concurrent administration of 2 bladder cancer promoters, Na-Sac plus Na-AsA, resulted in increases of carcinoma, papilloma, and preneoplastic hyperplasia greater than the simple sum of the promoting effects of the two promoters administered separately. Therefore, the data clearly show synergism of their promoting actions. Synergistic effects of carcinogens on tumor induction are well documented for many organs (1–4). In bladder carcinogenesis, we reported the synergistic interaction of 4 carcinogens in the induction of tumors (5). Also, we detected synergistic effects of bladder promoters using doses of half of those used to exert promoting activity of 2-stage bladder carcinogenesis when administered also (18). The present study showed synergism of bladder promoters administered at full doses. Since the magnitude of the increases of urinary pH and Na⁺ concentration following Na-Sac plus Na-AsA treatment was essentially not different from that following Na-Sac or Na-AsA alone, the enhancement of bladder carcinogenesis promotion induced by these 2 promoters together may depend on interactions of saccharin and ascorbate. Moreover, since Na-Sac plus AsA did not show a synergistic action of bladder carcinogenesis promotion, it is apparent that the interaction of the activities of saccharin and ascorbate is exerted only with conditions of increased urinary pH and Na⁺ concentration. With regard to the mechanism of synergism of promotion, further studies are required.

In previous experiments (10, 12), we reported that AsA acts

as a copromoter (an amplifier) under conditions of increased urinary pH and Na⁺ concentration. However, in the present study simultaneous treatment with AsA reduced the promoting activity of Na-Sac on bladder carcinogenesis and did not cause the elevation of urinary pH, although the urinary Na⁺ concentration was increased. These results are consistent with our previous finding that the addition of NH₄Cl reduced the promoting activity of Na-AsA in bladder carcinogenesis by reducing urinary pH, despite the urinary Na⁺ concentration being increased (12). Therefore, we have confirmed that the activity of AsA on amplification of promotion is exerted only under conditions of increased urinary pH. By lowering urinary pH, AsA acted as an antipromoter of bladder carcinogenesis in the present study.

Kakizoe *et al.* (19) found that many carcinogens and promoters of the bladder could maintain the state of increased concanavalin A agglutination of carcinogen-treated rat bladder cells. In addition, AsA was found to inhibit the agglutination of the bladder cells induced by Na-Sac, suggesting possible antipromoting activity for bladder carcinogenesis (20). These studies suggest that the activity of AsA as a copromoter or antipromoter is not a property of the chemical itself, but rather by its influence on urinary parameters.

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