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Inorganic Alkalizers and Acidifiers under Conditions of High Urinary Na^+ or K^+ on Cell Proliferation and Two-stage Carcinogenesis in the Rat Bladder

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Effects of alkalizers and acidifiers on bladder cell proliferation and two-stage carcinogenesis were investigated under conditions of high urinary Na^+ or K^+. Animals were given 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in their drinking water for 4 weeks and then received Na_3PO_4, NaH_2PO_4, NaCl, NaH_2PO_4+NaCl, K_3PO_4, KH_2PO_4, KCl, KH_2PO_4+KCl or no chemical supplement in the diet from weeks 5 to 8 and from weeks 12 to 20. During weeks 9 to 11, the rats were fed 3% uracil in their diet for acceleration of promotion. Na_3PO_4 or K_3PO_4 induced marked natriuresis or kaluresis and alkalinuria associated with strong promoting potential for bladder carcinogenesis. NaH_2PO_4 induced moderate natriuresis and aciduria and exhibited weak promoting activity. NaH_2PO_4+NaCl or KH_2PO_4+KCl caused marked increase in the respective cation levels and aciduria with elevation of promotion as compared to NaH_2PO_4 or KH_2PO_4 alone. NaCl or KCl induced moderate natriuresis or kaluresis and did not alter urinary pH. NaCl but not KCl also exerted weak promoting activity for bladder carcinogenesis. Increased DNA synthesis after test chemical exposure for 8 weeks and morphological alterations observed by scanning electron microscopy in the bladder epithelium were only quantitatively linked with promoting activity in the Na_3PO_4 case. With the other treatments no clear correlation between early cell proliferation and promotion potential was apparent. The present results suggest that although elevation in urinary Na^+ or K^+ level may be an essential factor for promotion of rat bladder carcinogenesis, the action of these cations may depend strongly on urinary alkalinity.

Key words: Sodium phosphate salt — Potassium phosphate salt — Urinary cation — pH — Bladder tumor promotion

A number of causal factors have been identified for human bladder cancer. In recent years, epidemiologic investigations have suggested that non-occupational factors may play a more crucial role in bladder carcinogenesis than occupational factors.[1-3] In particular, dietary factors such as artificial sweeteners, coffee, alcoholic beverages and contaminating nitrosamines have been suggested to be of importance.[4-6] Experimental animal studies have also indicated that many food additives can exert significant effects on rat bladder carcinogenesis.[7-11]

There have been many experimental reports on participation of urine itself or its constituents in cell proliferation or tumor promotion of the bladder in rats. Namely, alkalinity, cations (Na^+ or K^+), $MgNH_4PO_4$ crystals, growth factors, diuresis and others have been implicated.[8, 12-25] We focused on Na^+, K^+ and pH in the present study. Na_3PO_4 and K_3PO_4 cause alkalinuria, while NaH_2PO_4 and KH_2PO_4 induce aciduria, both causing natriuresis or kaluresis to a degree dependent on the contents of Na or K in the applied regimens. The present experiment was performed using these phosphate salts as food additives, in order to clarify further their promoting effects under conditions of different degrees of urinary natriuresis or kaluresis in combination with alkalinity or acidity. In addition, since tumor promotion might be due to an early proliferative response,[22, 26] their influence on bladder cell replication was also studied after administration of similar doses of the same test chemicals for 8 weeks.

MATERIALS AND METHODS

Animals A total of 220 male 6-week-old Fischer 344 rats (Charles River Japan, Inc., Atsugi) were used. They were housed five per plastic cage on hardwood chip bedding in an environment-controlled room, maintained at 22±2°C and 60±10% relative humidity and artificially illuminated for 12 h each day.

Chemicals BBN[4] was purchased from Tokyo Kasei Kogyo Co. (Tokyo), uracil from Yamasa Shoyu Co.

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[4] Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; BrdU, 5-bromo-2′-deoxyuridine; PN hyperplasia, papillary or nodular hyperplasia; SEM, scanning electron microscopy; TCC, transitional cell carcinoma.

(Choshi), and trisodium phosphate (Na_3PO_4) from Katayama Chemical Industries (Osaka). Monosodium phosphate (NaH_2PO_4), tripotassium phosphate (K_3PO_4) and monopotassium phosphate (KH_2PO_4) were from Nakalai Tesque, Inc. (Kyoto). Sodium chloride (NaCl) and potassium chloride (KCl) were from Wako Pure Chemical Industries (Osaka). All sodium or potassium salts used in this experiment were anhydrous.

Two-stage bladder carcinogenesis study The experimental protocol followed is shown in Fig. 1. The animals were randomly divided into 17 groups of 15 or 5 rats each. For the first 4 weeks, rats were given drinking water containing 0.05% BBN (groups 1 to 9) or normal tap water without BBN (groups 10 to 17). During weeks 5 to 8 (4 weeks) and weeks 12 to 20 (9 weeks), they received the following chemical supplements (15 rats each in groups 1 to 8 and 5 rats each in groups 10 to 17) in basal diet (Oriental MF, Oriental Yeast Co., Tokyo) or the basal diet alone as a control group (15 rats in group 9): 3% Na_3PO_4; 3% NaH_2PO_4; 1.7% NaCl; 3% NaH_2PO_4+1.7% NaCl; 3% K_3PO_4; 3% KH_2PO_4; 1.5% KCl; 3% KH_2PO_4+1.5% KCl. The dose levels of sodium or potassium phosphate salts were chosen on the basis of results regarding urinary pH and cation levels and toxicity from a preliminary study (unpublished data). The dose levels of NaCl or KCl were respectively determined to give the same relative weights of Na or K when combined with NaH_2PO_4 or KH_2PO_4 as with Na_3PO_4 or K_3PO_4 alone. For acceleration of transitional cell proliferation, the rats of all groups received 3% uracil in the diet during weeks 9 to 11 (3 weeks). Animals were observed daily to assess general health and body weight, and food and water consumptions were measured weekly.

Urinalysis was performed on five rats each of all BBN-treated groups (groups 1–9) at weeks 14 and 20. Urinary pH values were determined with a pH meter (model F-8DP, Hitachi-Horiba, Tokyo) using freshly voided urine specimens at 08.00 h. In addition, rats were placed in individual metabolic cages without food and water for collection of urine samples over a 4-h period (09.00–13.00 h), for subsequent measurement of osmolality (by freezing-point depression using Osmett A, Precision Systems Inc., MA, USA) and mineral concentrations (Na^+, K^+, Ca^{2+}, Mg^{2+}, PO_4^{3-}). Remaining urinary samples were centrifuged and examined microscopically for sediment.

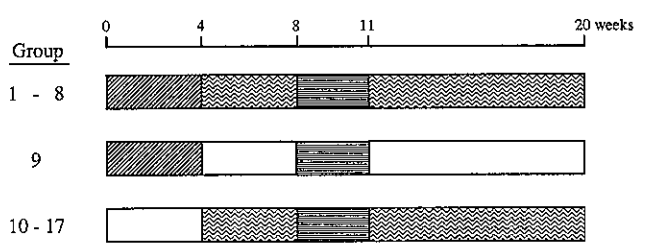

Fig. 1. Experimental protocol for investigation of effects of urinary cations and pH on rat two-stage bladder carcinogenesis. ▨: 0.05% BBN in drinking water. ▭: 3% uracil in diet. ▩: Test chemicals in diet as follows: sodium salts; 3% Na_3PO_4, 3% NaH_2PO_4, 1.7% NaCl, 3% NaH_2PO_4+1.7% NaCl, and potassium salts; 3% K_3PO_4, 3% KH_2PO_4, 1.5% KCl, 3% KH_2PO_4+1.5% KCl.

At week 20, the animals were killed by exsanguination under ether anesthesia. The urinary bladders were ligated at the neck, inflated by intraluminal injection of 10% phosphate-buffered formalin, and excised. After fixation, bladders were bisected sagittally and excess moisture was absorbed with filter papers. After being weighed, the bladders were cut into eight strips, embedded in paraffin, sectioned, and stained routinely with hematoxylin and eosin.

Cell proliferation study This study was conducted to investigate the effects of test chemicals alone on bladder epithelium. Rats were randomly allocated to eight treated groups and one control group comprising five rats each. They were given basal diet alone or diet supplemented with sodium or potassium salts at the same dietary levels as for the two-stage study. Sequential urinalyses (pH and mineral ion concentrations) were conducted at weeks 2, 4 and 8. At the termination at week 8, all rats were injected i.p. with 100 mg/kg body weight of BrdU (Sigma Chemical Co., MO, USA). Circadian rhythms have been demonstrated in a number of cell cycle components.[27–29] Therefore, in order to avoid any variance between groups, the animals were sequentially killed so that one rat from each of the groups was simultaneously killed by exsanguination under ether anesthesia 1 h after BrdU injection. This process was repeated until all the animals had been processed. Bladders were inflated with ice-cold 10% formalin in 0.1 *M* phosphate buffer (pH 7.4), removed quickly, and immersed in the fixative. After 2 h fixation, the bladders were divided in half longitudinally. One half was processed for both histopathology examination and immunohistochemical BrdU staining[30] (Vectastain ABC Kit, Vector Laboratories Inc., CA, USA) to assess DNA synthesis; the numbers of S phase cells incorporating BrdU into DNA per 1,000 cells were counted. The other half was used for detection of morphological changes at the surface of superficial epithelial cells using SEM (Hitachi S-450, Hitachi Co., Ltd., Tokyo).

Statistical analysis The significance of differences between groups in body and bladder weights, urinalysis data, levels of DNA synthesis and numbers of urinary bladder lesions was analyzed by using Student's *t* test

according to Welch (in cases of insufficient homogeneity of variance). The significance of differences in lesion incidences between different groups was examined by using Fisher's exact probability test.

RESULTS

Two-stage bladder carcinogenesis study

Clinical observation did not reveal any abnormalities or differences among the groups, and no deaths occurred during the treatment period. Data on body and urinary bladder weights, and food and water consumption in BBN-treated groups are presented in Table I. Significant decreases in final body weights were observed for the Na_3PO_4 (group 1) and NaH_2PO_4+NaCl (group 4) groups when compared to the control (group 9). There was no apparent difference in food consumption among the groups. Water consumption showed a tendency for increase in rats receiving Na_3PO_4, NaCl, NaH_2PO_4+NaCl, K_3PO_4 or KH_2PO_4+KCl. Absolute or relative bladder weights were significantly increased in all treated groups with the exceptions of the KH_2PO_4 and KCl alone groups.

Urinalysis The results of urinalysis at week 20 are shown in Table II. Urine osmolality showed no statistically significant variation among the groups. Urinary pH was significantly elevated in groups given Na_3PO_4 or K_3PO_4, while NaH_2PO_4 alone or with NaCl, and KH_2PO_4 alone or with KCl caused a drop in pH. Increased levels of urinary Na^+, K^+ or PO_4^{3-} were generally in line with those expected for the individual dosing regimens. In addition, a decrease in Ca^{2+} was observed in rats given

Table I. Average Final Body and Bladder Weights, and Food and Water Consumption Data (Weeks 5–36)[a]

Group	Treatment		No. of rats	Final body weight (g)	Bladder weights		Food consumption (g/rat/day)	Water consumption (g/rat/day)
	BBN/ Uracil	Test chemicals			Absolute (g)	Relative (%)		
1	+	Na_3PO_4	15	354±17*	0.328±0.072**	0.093±0.022**	14.8	26.1
2	+	NaH_2PO_4	15	364±25	0.227±0.068**	0.062±0.019**	14.7	24.7
3	+	NaCl	15	372±17	0.184±0.022**	0.049±0.007**	15.3	25.8
4	+	NaH_2PO_4+NaCl	15	354±14*	0.226±0.044**	0.064±0.013**	15.1	26.8
5	+	K_3PO_4	15	370±28	0.221±0.050**	0.060±0.013**	15.2	25.4
6	+	KH_2PO_4	15	379±16	0.168±0.031*	0.044±0.010	15.8	24.1
7	+	KCl	15	365±16	0.161±0.031	0.044±0.010	14.8	24.8
8	+	KH_2PO_4+KCl	15	368±16	0.170±0.022**	0.047±0.008**	15.8	26.1
9	+	—	15	370±17	0.142±0.017	0.038±0.005	14.9	23.6

a) Values are mean±SD.
* $P<0.05$; ** $P<0.01$ compared with control values (group 9).

Table II. Urinary Characteristics of Rats Treated with Na or K Phosphate Salts during Two-stage Bladder Carcinogenesis[a]

Treatment		No. of rats	Urine osmolality (mOsm/kg H_2O)	Urine pH	Urine level (mEq/liter) of:			Urine level (mg/dl) of:		Crystals[b]
BBN/ Uracil	Chemical				Na	K	Ca	P	Mg	
+	Na_3PO_4	5	1281±64	7.1±0.2*	342±19**	128±17	4.1±1.8	120±41**	16±8	+++
+	NaH_2PO_4	5	1598±170	6.0±0.1**	200±42**	142±71	5.2±0.5	165±53**	19±5	++
+	NaCl	5	1639±319	6.8±0.3	232±72**	149±31	10.6±3.7	11±9	38±8	+
+	NaH_2PO_4+NaCl	5	1450±146	6.0±0.1**	404±71**	133±33	4.9±2.1	172±88*	14±6	++
+	K_3PO_4	5	1485±240	7.2±0.1**	53±25	352±108**	3.6±0.9**	160±65**	20±8	+++
+	KH_2PO_4	5	1535±313	6.0±0.2**	66±41	265±37**	2.8±0.6**	309±167*	21±8	+
+	KCl	5	1386±373	6.8±0.2	66±39	273±57**	8.7±3.5	17±16	56±17*	+
+	KH_2PO_4+KCl	5	1118±312	6.2±0.1*	72±30	378±35**	3.5±0.8**	383±66**	19±3	+
+	—	5	1429±297	6.7±0.3	45±10	111±34	7.2±3.0	8±6	22±12	+

a) Values are mean±SD.
b) Grading (mean of group): ±, trace; +, slight; ++, moderate; +++, severe.
* $P<0.05$; ** $P<0.01$ compared with control values.

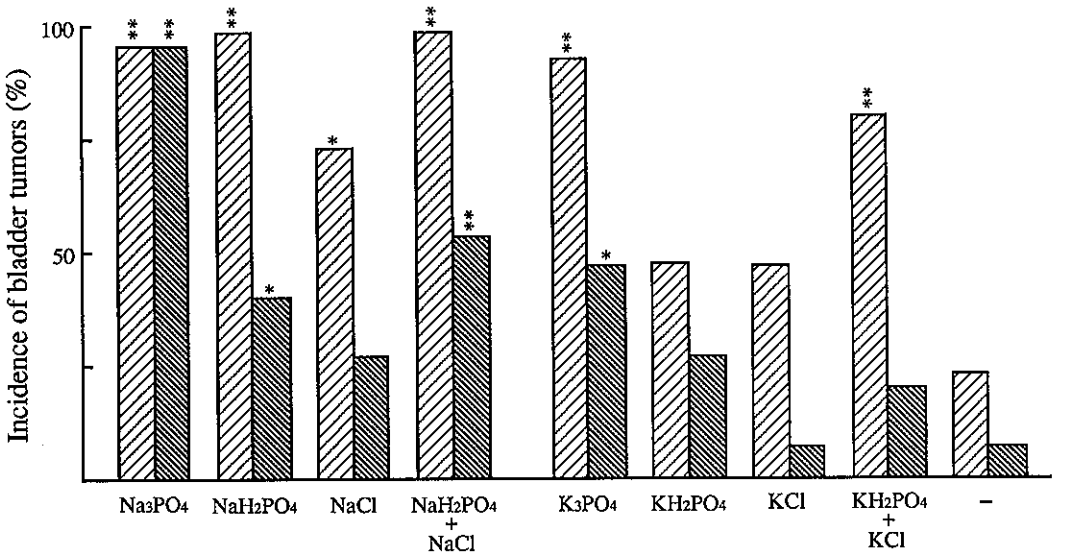

Fig. 2. Incidence of bladder tumors (▨: papilloma; ▩: TCC) in rats treated with Na or K phosphate salts on the two-stage bladder carcinogenesis regime. Significantly different from control values at $P<0.05$ (*) or $P<0.01$ (**).

Table III. Quantitation of Urinary Bladder Lesions in Rats Treated with Na or K Phosphate Salts during Two-stage Bladder Carcinogenesis

Group	Treatment		No. of rats	Numbers of preneoplastic or neoplastic lesions per rat[a]		
	BBN/ Uracil	Test chemicals		PN hyperplasia	Papilloma	TCC
1	+	Na_3PO_4	15	36.1 ± 12.0^{c}	9.8 ± 4.1^{c}	2.5 ± 1.5^{c}
2	+	NaH_2PO_4	15	15.3 ± 5.9^{c}	3.5 ± 1.5^{c}	0.4 ± 0.5
3	+	NaCl	15	14.5 ± 8.1^{b}	1.7 ± 1.7^{b}	0.3 ± 0.5
4	+	NaH_2PO_4+NaCl	15	$26.0\pm10.8^{c,d}$	$7.0\pm3.1^{c,d}$	0.9 ± 1.0^{b}
5	+	K_3PO_4	15	21.0 ± 6.1^{c}	4.0 ± 2.8^{c}	0.7 ± 0.8^{c}
6	+	KH_2PO_4	15	10.1 ± 4.7	1.2 ± 1.5	0.3 ± 0.4
7	+	KCl	15	8.3 ± 4.7	0.6 ± 0.7	0.1 ± 0.3
8	+	KH_2PO_4+KCl	15	$19.5\pm8.1^{c,d}$	1.9 ± 1.4^{c}	0.3 ± 0.3
9	+	—	15	9.3 ± 4.7	0.4 ± 0.6	0.1 ± 0.3

a) Numbers of bladder lesions per rat are expressed as mean±SD.
b) $P<0.05$; *c*) $P<0.01$ compared with control values (group 9).
d) $P<0.01$ compared with NaH_2PO_4 (group 2) or KH_2PO_4 (group 6).

phosphate salts, this being significant in the groups given K phosphate salts. The phosphate-associated reduction in urinary Ca^{2+} is probably due to formation of non-absorbable $CaPO_4$ salts in the lumen of the intestine.[31, 32] Ingestion of Na_3PO_4, NaH_2PO_4, alone or with NaCl, and K_3PO_4 induced an increase of $MgNH_4PO_4$ crystals observed in urinary sediments. Results of urinalysis at week 14 showed similar trends.

Urinary bladder lesions Grossly, nodular lesions were observed in the bladder mucosa of rats initiated by BBN. The degree of lesion development in rats given Na or K phosphate salts was more severe than in controls. No stone formation was found in any of the groups. Incidences of urinary bladder tumors in BBN-initiated groups (groups 1–9) are illustrated in Fig. 2. The occurrence of PN hyperplasia in all BBN-initiated groups was 100% (data not shown). Significant increase in incidences of papilloma was observed in the Na_3PO_4, NaH_2PO_4, NaCl, NaH_2PO_4+NaCl, K_3PO_4, and KH_2PO_4+KCl groups. The incidences of TCC were significantly increased in the Na_3PO_4, NaH_2PO_4, NaH_2PO_4+NaCl, and K_3PO_4 groups. Quantitative data

Table IV. Morphological Findings in Bladder Epithelium of Rats Given Na or K Phosphate Salts for 8 Weeks[a)]

Test chemicals	No. of rats	LM finding		SEM finding					
		Simple hyperplasia		Pleomorphic microvilli		Short, uniform microvilli		Ropy or leafy microridges	
		No. (%)	S	No. (%)	S	No. (%)	S	No. (%)	S
Na_3PO_4	5	5 (100)**	++	3 (60)	++	4 (80)*	++	5 (100)**	+++
NaH_2PO_4	5	0	−	0	−	0	−	3 (60)	++
NaCl	5	0	−	0	−	0	−	0	−
NaH_2PO_4+NaCl	5	0	−	0	−	2 (40)	+	3 (60)	++
K_3PO_4	5	0	−	0	−	2 (40)	+	2 (40)	+
KH_2PO_4	5	0	−	0	−	0	−	1 (20)	+
KCl	5	0	−	0	−	0	−	0	−
KH_2PO_4+KCl	5	0	−	0	−	0	−	1 (20)	+
—	5	0	−	0	−	0	−	1 (20)	+

LM, light microscopy; SEM, scanning electron microscopy; S, severity.
a) Grading of lesions (mean of group): −, no change; +, slight, ++, moderate, +++, severe.
* $P<0.05$; ** $P<0.01$ compared with control values.

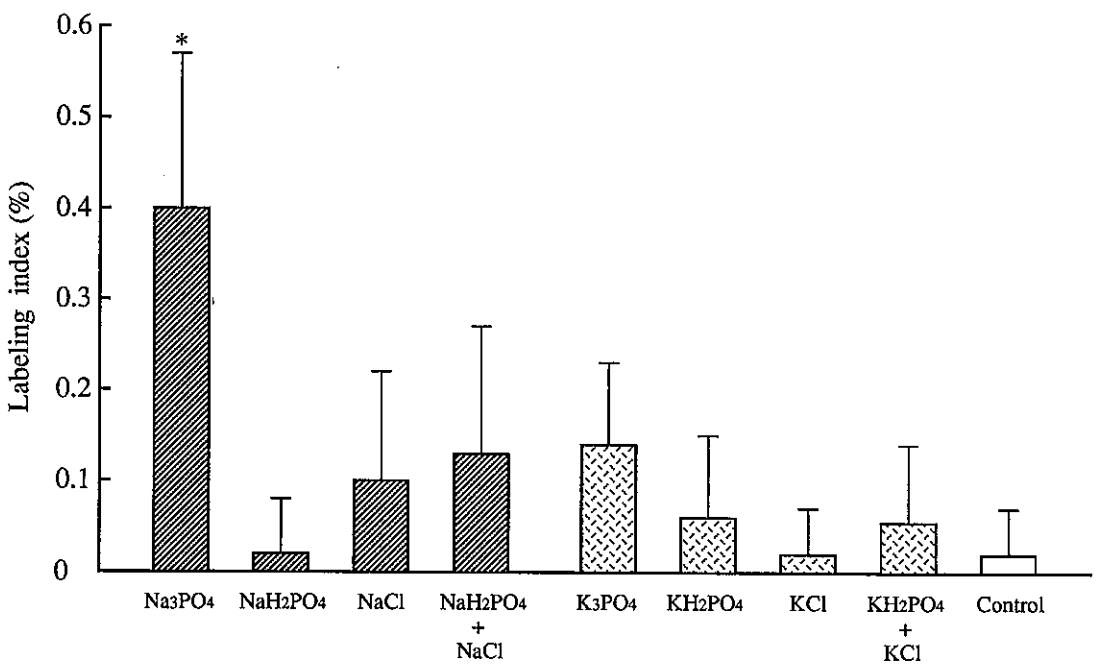

Fig. 3. Labeling index (%) values for bladder epithelium of rats treated with Na phosphate salts (▨) or K phosphate salts (▩) for 8 weeks. Data presented are mean±SD. Significantly different from control values at $P<0.05$ (*).

regarding numbers of preneoplastic or neoplastic lesions of the bladder are shown in Table III. Numbers of PN hyperplasia per rat were significantly increased in all treated groups except for the KH_2PO_4 or KCl groups, as compared to the control (group 9). In addition, treatment with NaH_2PO_4+NaCl or KH_2PO_4+KCl resulted in an elevation of the numbers per rat as compared to treatment with NaH_2PO_4 or KH_2PO_4 alone, respectively. Numbers of papillomas per rat were significantly increased in all treated groups, with the exception of the KH_2PO_4 and KCl groups, in comparison with the control. The numbers of papillomas per rat in the NaH_2PO_4+NaCl group were significantly higher than values for the NaH_2PO_4 group. With respect to TCC,

numbers per rat were significantly increased in the Na_3PO_4, NaH_2PO_4 and K_3PO_4 groups. In groups receiving test chemicals in sequential combination with uracil (without BBN initiation) (groups 10–17), the majority of the animals had simple hyperplasia. PN hyperplasia was observed for all cases (5/5) in the Na_3PO_4 group only (data not shown).

Eight-week cell proliferation study

Urinary pH and Na^+ or K^+ Consistently, urinary pH was increased in rats receiving Na_3PO_4 or K_3PO_4, whereas it was decreased in rats given NaH_2PO_4 or KH_2PO_4, independent of combination with NaCl or KCl. An increase in excreted Na^+ or K^+ was observed in association with the dosing regimens throughout the study (data not shown).

Morphological alterations Morphological findings are summarized in Table IV. Simple hyperplasia, consisting of focal/multifocal thickening of the epithelium with four layers of epithelial cells, was observed by light microscopy in all animals in the Na_3PO_4 group only. Under the SEM, normal bladder epithelium presents a flat appearance and is composed of large polygonal cells of relatively uniform size and shape whose luminal surfaces are covered with a complex network of microridges. Epithelial surface alterations of the bladder, such as pleomorphic microvilli, uniform or short microvilli and ropy or leafy microridges, were observed by SEM and were classified as described previously.[33] These epithelial changes, and especially microvilli, are regarded as being indicative of cell proliferation. Surface cell changes were noted in all treated groups with the exception of those receiving NaCl or KCl alone. The most severe alterations were observed in rats given Na_3PO_4. In particular, pleomorphic microvilli were limited to this group. Short or uniform microvilli were observed in rats receiving Na_3PO_4, NaH_2PO_4+NaCl or K_3PO_4. Other epithelial alterations were noted in all groups including the control group, with the exception of the NaCl or KCl groups. No differences in bladder epithelial changes between groups given NaH_2PO_4 alone or with NaCl or KH_2PO_4 alone or with KCl could be distinguished by SEM.

DNA synthesis Bladder epithelial labeling indices (%) as assessed by incorporation of BrdU into DNA in treated and control rats are shown in Fig. 3. Significant elevation in labeling indices as compared to the control was only seen in the Na_3PO_4 group. There were no differences in labeling indices between the other treated groups and the control.

DISCUSSION

The results of the present study can be summarized as follows. (i) Among the Na phosphate or the K phosphate treatments, promotion of bladder tumor development was strongest in the Na_3PO_4 or the K_3PO_4 group, respectively, both regimens inducing urinary conditions of markedly increased Na^+ or K^+ with alkalinuria. (ii) NaH_2PO_4 exerted promoting activity, associated with moderate increase in urinary Na^+ and aciduria. (iii) Simultaneous administration of NaCl with NaH_2PO_4 strongly enhanced the promotion of bladder carcinogenesis as well as bringing about a marked increase in urinary Na^+ with aciduria. However, KCl in combination with KH_2PO_4 enhanced preneoplasia development only under conditions of marked kaluresis with aciduria. (iv) NaCl itself exhibited weak promoting activity, inducing a moderate increase in urinary Na^+ without alteration of urinary pH. On the other hand, KCl did not promote bladder carcinogenesis while causing a moderate increase in urinary K^+ with normal urinary pH. (v) Treatment with Na_3PO_4, causing induction of natriuresis and alkalinuria, brought about an early cell proliferation in the bladder which directly correlated with promoting activity. However, the other treatments did not show any such clear association.

It has been well documented that high concentrations of Na^+ or K^+ and alkalinuria may be contributory factors for promotion of urinary bladder tumor development. For example, recent *in vivo* studies demonstrated that various organic sodium salts exert significant promoting activity on two-stage bladder carcinogenesis in rats, although the parent acid compounds proved inactive.[8, 34, 35] It has further been shown that $NaHCO_3$ or K_2CO_3, but not $MgCO_3$ or $CaCO_3$, can similarly enhance bladder carcinogenesis.[8, 21, 36, 37] Several studies have demonstrated that NH_4Cl (used as a urine acidifier) inhibits the promoting activity of sodium L-ascorbate[17] and sodium saccharin[8, 38] and reduces the induction of bladder cancer by 4-ethylsulfonylnaphthalene-1-sulfonamide[39] or sodium *o*-phenylphenate.[40] Although acetazolamide, $CaCO_3$ or $MgCO_3$ induce alkalinuria, they have no promoting action for rat bladder.[10, 37] The simplest of the sodium salts, NaCl, at a massive dose level (10%) exerts a weak promoting effect on two-stage bladder carcinogenesis.[41] While 1% and 5% levels of NaCl have been reported to lack promoting potential,[41, 42] recent studies demonstrated 1.34% or 2.34% NaCl also to exhibit weak tumor promoting activity in rat bladder carcinogenesis.[8, 21] NaCl ingestion causes natriuresis but not alkalinuria. In the present study, 1.7% NaCl also weakly enhanced tumor development. In a recent study using a simple initiation-promotion protocol, the urine alkalizer 1.4% Na_3PO_4 or 1.0% K_3PO_4 enhanced preneoplastic lesion development, while 2.0% NaH_2PO_4 or 2.5% KH_2PO_4, a urine acidifier, did not exert any promoting activity. All of these chemicals induced moderate natriuresis (190–300 mEq/liter) or kaluresis (250–370 mEq/liter), and thus tumor promotion apparently de-

pends on high urinary pH under conditions of either moderate natriuresis or kaluresis.[43] The results gained using the present highly sensitive bladder carcinogenesis model showed that a moderate increase in urinary Na^+ levels is enough to exert weak bladder tumor promotion, this difference being possibly due to the accelerating effect of uracil. However, the present modal also showed that strong tumor promotion does require alkaline conditions. Although a critical level of K^+ in the urine has also been considered to be an essential factor for bladder tumor promotion, this cation may strongly depend on urine alkalinity for its action.

Many *in vitro* investigations have found a positive correlation between cation (Na^+ or K^+) influx and stimulation of DNA synthesis and cell proliferation. Rozengurt and Mendoza[44] reported that DNA synthesis stimulated by serum in quiescent cultures of 3T3 cells depends on the concentrations of Na^+ and K^+ in the nutrient medium. They thus provided direct evidence for a link between monovalent cation transport and cell proliferation. While *in vitro* studies also demonstrated both Na^+ and K^+ to be possible triggers of cell proliferation, the differences in effects on normal and tumor cells might indicate basic differences in the mechanisms of action of these two cations. Cameron *et al.*[45] reported that high intracellular sodium concentrations are associated with mitogenesis, whereas an elevated intracellular concentration of potassium appears to be related to maintenance of a high rate of mitotic activity in non-tumor cells. However, it is not necessary for the maintenance of a high rate of mitotic activity in tumor cells. In addition, it was previously reported that bladder epithelia of rats treated with the bladder tumor promoter sodium saccharin show an elevation in membrane potential which is associated with the cellular Na/K ion pump.[46] Similarly, a rise in intracellular pH is believed to play a fundamental role in cell growth,[47] and proliferation in a variety of cell lines is extremely sensitive to changes in extracellular pH.[48, 49] Thus, changes in urine constituents could influence normal cellular regulation and the activity of a variety of enzymes, proteins, and metabolic pathways. This might be expected to be reflected in morphologically altered rat bladder epithelium. In fact, sodium or potassium salt types of bladder tumor promoters induce pleomorphic microvilli on superficial cells of the bladder in rats.[18, 22, 33, 50, 51] The observed pleomorphic microvilli on superficial epithelial cells are characteristic of basal cells in urinary bladder,[51] suggesting abnormality of cell differentiation,[52] indicating a proliferative response,[22, 23, 50] and resulting in tumor promotion.

In conclusion, under the conditions of the present study, elevation in urinary Na^+ or K^+ levels was found to be of essential significance to promotion of bladder carcinogenesis, but the action of these cations may depend strongly on urinary alkalinity.

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