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Effects of Promoters on *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine-Induced Urinary Bladder Carcinogenesis in the Rat

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It has been shown that the occurrence of the preneoplastic lesion, papillary or nodular hyperplasia (PN hyperplasia) in rat urinary bladder induced by carcinogens is correlated with that of cancer. Therefore, the promoting effects of chemicals in two-stage bladder carcinogenesis were judged by measuring their ability to induce PN hyperplasia in rats. Male rats were given *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) for 4 weeks and then one of 16 test chemicals for 32 to 34 weeks. Saccharin, ascorbate, DL-tryptophan, allopurinol, and diphenyl promoted development of PN hyperplasia. The dose-response of the promoters were examined in both sexes of rats by administration of saccharin at doses of 0.04, 0.2, 1.0 and 5.0% for 32 weeks after BBN treatment. The occurrence of PN hyperplasia was significantly increased in the group given 5% saccharin. Dose-response curves showed enhanced hyperplastic responses in both sexes given 0.2 to 5% saccharin.

The organ specificities of promoters were studied in rats initiated with BBN or 2-acetylaminofluorene (2-AAF) followed by phenobarbital or saccharin for 32 weeks. Phenobarbital greatly enhanced hepatocarcinogenesis. Saccharin significantly enhanced the occurrence of both BBN-induced and 2-AAF-induced PN hyperplasia. However, there was no effect of phenobarbital on the urinary bladder or of saccharin on the liver.

The rats showed a strain difference in susceptibility of the urinary bladder to saccharin; ACI rats were most susceptible and Sprague Dawley rats were most resistant to saccharin.

The membrane potential of superficial epithelial cells in the urinary bladder of rats treated with saccharin was measured with an intracellular microelectrode and found to be higher than that of controls.

Introduction

The two-stage process of chemical carcinogenesis, initiation and promotion, was initially proposed from studies on mouse skin and has subsequently been demonstrated in other organs, such as the liver, colon and urinary bladder (*1-3*). The two-stage process of urinary bladder carcinogenesis has been demonstrated in rats using single intravesical instillation of methylnitrosourea (*4*) or initiation by *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) (*5*), followed by treatment with saccharin. In the present studies we examined the promoting effects of various chemicals on urinary bladder carcinogenesis in rats initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) and evaluated the *in vivo* biological characteristics of tumor promoters in urinary bladder carcinogenesis using saccharin as a typical promoter. Effects were mainly judged by measuring the formation of the preneoplastic lesion and papillary and nodular hyperplasia of the urinary bladder.

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The Preneoplastic Lesion and Histological Observation

BBN is a potent carcinogen in the urinary bladder of animals. There have been many reports on the carcinogenicity of BBN in rat urinary bladder (*6-8*). Histological lesions in the urinary bladder of rats induced by BBN were classified into four types, simple hyperplasia, PN hyperplasia, papilloma and cancer (*9*). As shown in Figure 1, PN hyperplasia of the urinary bladder in rats treated with 0.05% BBN developed before the induction of papilloma or cancer (*6*). The incidence of PN hyperplasia in the uri-

nary bladder of rats treated with various doses of BBN for different periods is shown graphically in Figure 2 (7). A high dose of BBN induced a high incidence of PN hyperplasia in a short period. Dose-response relationships were observed for the induction of PN hyperplasia, the dose of BBN, and the period of BBN treatment. Moreover, a good correlation was found between the inductions of PN hyperplasia and cancer with different doses of BBN. Thus, it is clear that PN hyperplasia is a preneoplastic lesion of rat urinary bladder, and can be used as a marker in studies on the 2-stage process of urinary bladder carcinogenesis.

For histological examination of the urinary bladder, rats were killed with ether, 10% phosphate-buffered formalin (pH 7.4) was injected into the urinary bladder through the urethra, and the urinary bladder was then divided in half sagitally. Each half was cut into four to six strips, which were embeddee in paraffin. Tissue sections were stained with hematoxylin and eosin. In quantitative analyses, the numbers of lesions were counted under a light microscope, the total length of the basement membrane was measured with a color video image processor (VIP-21 or 21C; Olympus- Ikegami Tsushin Co., Tokyo, Japan), and the results were expressed as the number of lesions per 10 cm of basement membrane.

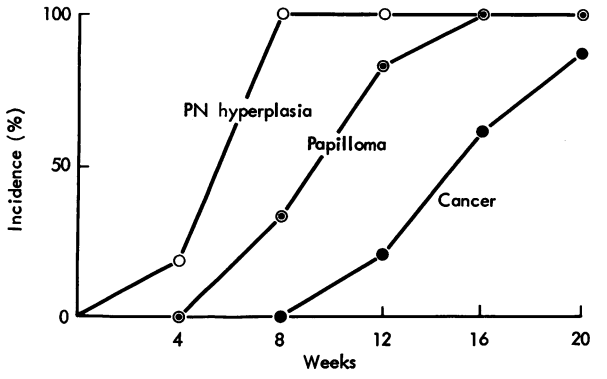

FIGURE 1. Incidence of urinary bladder lesions in rats given 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

Promoting Effects of Various Chemicals in Rat Urinary Bladder Carcinogenesis Initiated by BBN

Saccharin and tryptophan are known to act as promoters after initiation of the urinary bladder with carcinogens (*4,5,10*). Various chemicals are present in the environment and a high proportion of human cancers is attributable to environmental factors. Thus since some of the environmental chemicals to which humans are exposed may promote urinary bladder carcinogenesis, special consideration should be paid to evaluation of their promoting activities. Accordingly, the following experiment was carried out. Carcinogenesis of the urinary bladder was initiated in male 6-week-old F344 or Wistar rats by adding 0.01% BBN to their drinking water for 4 weeks, and then the animals were given a test chemical to examine its promoting activity. The

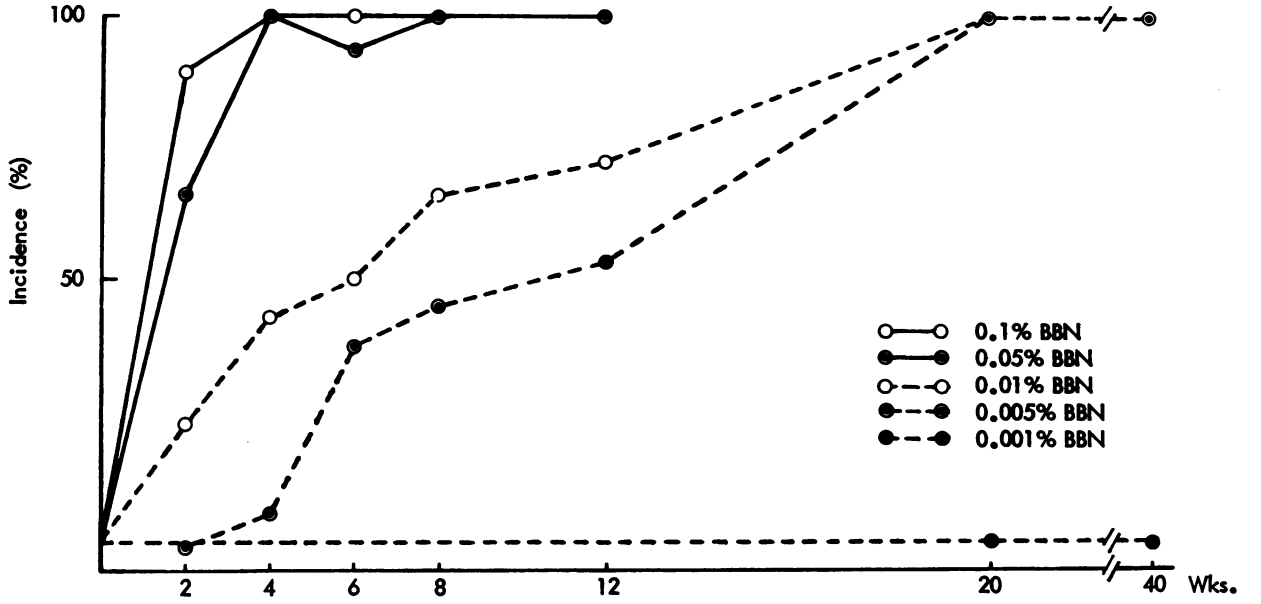

FIGURE 2. Incidence of PN hyperplasia in the urinary bladder of rats given *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN).

chemicals tested, their dose levels and the observation periods used are shown in Figure 3.

The incidence and number of PN hyperplasia per 10 cm of basement membrane are shown in Table 1. Administration of 5% saccharin, but not 0.5% saccharin, significantly increased the incidence and number of PN hyperplasia. This finding could be related to the induction of cancers in rat urinary bladder by high levels of saccharin. Sodium ascorbate, DL-tryptophan, allopurinol and diphenyl also increased the numbers of PN hyperplasia significantly, but other test chemicals, such as acetazolamide, quercetin, cyclamate, *o*-phenylphenol, and caffeine did not. Results on saccharin and tryptophan are consistent with previous findings (*5*) and suggest that sodium ascorbate, allopurinol and diphenyl have promoting activities in urinary bladder carcinogenesis in rats. No correlation was found between the number of crystals in the urine sediment and promotion of preneoplastic lesion, but further studies are required on this problem.

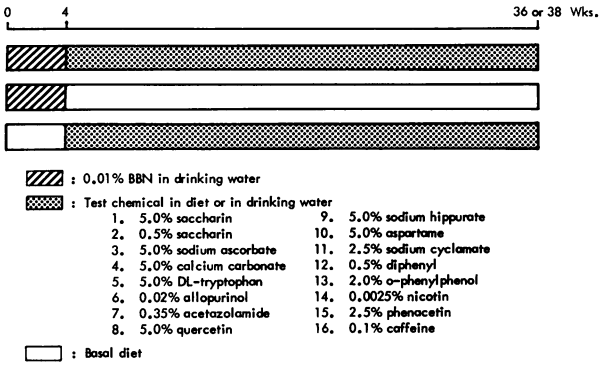

FIGURE 3. Experimental protocol for the evaluation of promoting activity of various chemicals in two-stage bladder carcinogenesis of rats initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN).

Dose-Response Relationship of Saccharin as a Promoter of Urinary Bladder Carcinogenesis

In general, chemical carcinogens show dose-response relation for both tumor induction and the length of the induction period (*8,11*). Dose-response studies have been undertaken with the use of saccharin as a promoter for preneoplastic lesions in the urinary bladder (*12*). F344 rats of both sexes were initiated by treatment with 0.01% BBN in their drinking water for 4 weeks and then given diets containing saccharin at concentrations of 5, 1, 0.2, 0.04 and 0% for 32 weeks. The animals were killed at the end of week 36. The incidences and average numbers of PN hyperplasia per 10 cm of basement

Table 1. Promotion effects of various chemicals on the induction of PN hyperplasia in rat urinary bladder initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN).[a]

		Observation			PN hyperplasia[c]	
Test Chemical	Administration route[b]	Rat strain	period, weeks	No. of rats	Incidence, %	No./10 cm of basement membrane
Saccharin (5.0%)	D	F344	38	30	25 (83.3)*	2.2 ± 2.7**
Saccharin (0.5%)	D	F344	38	30	14 (46.7)	1.0 ± 1.5
Sodium ascorbate	D	F344	38	29	19 (65.5)	2.0 ± 2.1**
Calcium carbonate	D	F344	38	30	14 (46.7)	0.7 ± 0.8
DL-Tryptophan	D	F344	38	30	16 (53.3)	1.5 ± 1.9***
Allopurinol	D	F344	38	28	16 (57.1)	1.9 ± 2.3**
Acetazolamide	D	F344	38	27	12 (44.1)	0.9 ± 1.5
Quercetin	D	F344	38	29	9 (31.0)	0.4 ± 0.6
Sodium hippurate	D	F344	38	30	11 (36.7)	0.6 ± 0.8
Aspartame	D	F344	36	28	8 (28.6)	0.5 ± 0.6
Sodium cyclamate	D	F344	36	30	17 (56.7)	0.7 ± 0.7
Diphenyl	D	F344	36	25	8 (32.0)	1.4 ± 0.8**
o-Phenylphenate	D	F344	36	30	8 (26.7)	1.1 ± 0.1
Nicotine	W	F344	36	30	10 (33.3)	0.4 ± 0.5
Phenacetin	D	Wistar	36	28	16 (57.1)	2.1 ± 3.4**
Caffeine	W	Wistar	36	28	12 (43.0)	0.6 ± 0.8
None	–	F344	38	60	27 (45.0)	0.6 ± 0.8
None	–	F344	36	28	11 (39.3)	0.7 ± 0.8
None	–	Wistar	36	23	6 (26.1)	0.5 ± 1.3

[a]The rats were initiated with 0.01% BBN in their drinking water for 4 weeks.
[b]D = in diet; W = in drinking water.
[c]Values are means ± SD.
***$p < 0.05$ compared with no test chemical.
**$p < 0.01$ compared with no test chemical.
*$p < 0.001$ compared with no test chemical.

membrane in males and females treated with saccharin after BBN are shown in Tables 2 and 3, respectively. The incidences in males with a dose of 5% saccharin and in females with doses of 5% and 1% were significantly higher than those with BBN alone. Plots of the incidence of PN hyperplasia against the dose of saccharin gave parabolic curves in both sexes (Fig. 4); those dose-response curves showed enhanced hyperplastic responses in both sexes given 0.2 to 5% saccharin. Administration of various doses of saccharin without BBN did not cause any changes in the urinary bladder of rats of either sex. Although no increase in the induction of papilloma or cancer by saccharin was noted in either sex after BBN administration, the results suggest that if the experimental period had been longer, a dose-response relationship for the induction of papilloma and cancer might have been observed. A similar dose-response relation for the effect of a promoter has been reported (*13*) for liver carcinogenesis initiated by 2-acetylaminofluorene (2-AAF).

Promoting Effects of Saccharin with Two Different Initiating Doses of BBN

It is generally accepted that initiation of chemical carcinogenesis is brought about by chemicals that are mutagenic in a short-term *in vitro* assay. Dose-related mutagenic effects have been reported for many chemical carcinogens, and *in vivo* dose-response relations of initiation activities have been studied in two-stage carcinogenesis in the skin and liver (*14,15*).

Table 2. Lesions of the urinary bladder in male rats initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) followed by different doses of saccharin.

Treatment[a]			PN hyperplasia[b]		Papilloma[b]	
BBN	Dose of saccharin %	No. of rats	Incidence, %	No./10 cm of basement membrane	Incidence, %	No./10 cm of basement membrane
+	5.0	29	24 (82.8)*	2.6 ± 2.2***	6 (20.7)	0.2 ± 0.4
+	1.0	30	19 (63.3)	1.2 ± 1.7	10 (33.3)	0.3 ± 0.4
+	0.2	30	14 (46.7)	0.9 ± 1.2	8 (26.7)	0.3 ± 0.5
+	0.04	30	12 (40.0)	0.6 ± 0.8	11 (36.7)	0.3 ± 0.4
+	0	28	11 (39.3)	0.7 ± 1.0	8 (28.6)	0.4 ± 0.7
−	5.0	28	0 −	0	0 −	0
−	1.0	28	0 −	0	0 −	0
−	0.2	30	0 −	0	0 −	0
−	0.04	29	0 −	0	0 −	0
−	0	29	0 −	0	0 −	0

[a]The rats were initiated with 0.01% of the drinking water for 4 weeks.
[b]Values are means ± SD.
***$p < 0.05$ compared with BBN alone.
*$p < 0.001$ compared with BBN alone.

Table 3. Lesions of the urinary bladder in female rats initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) followed by different doses of saccharin.

Treatment[a]			PN hyperplasia[b]		Papilloma[b]	
BBN	Dose of saccharin %	No. of rats	Incidence, %	No./10 cm of basement membrane	Incidence, %	No./10 cm of basement membrane
+	5.0	31	23 (74.2)*	2.9 ± 3.7**	1 (3.2)	0.1 ± 0.4
+	1.0	30	17 (56.7)***	1.4 ± 1.8	2 (6.7)	0.1 ± 0.3
+	0.2	31	14 (45.2)	1.4 ± 2.0	1 (3.2)	0.0 ± 0.2
+	0.04	31	11 (35.5)	1.0 ± 1.9	1 (3.2)	0.0 ± 0.2
+	0	29	9 (31.0)	0.7 ± 1.2	8 (3.4)	0.0 ± 0.2
−	5.0	32	0 −	0	0 −	0
−	1.0	30	0 −	0	0 −	0
−	0.2	31	0 −	0	0 −	0
−	0.04	31	0 −	0	0 −	0
−	0	30	0 −	0	0 −	0

[a]The rats were initiated with 0.01% BBN in their drinking water for 4 weeks.
[b]Values are means ± SD.
***$p < 0.05$ compared with BBN alone.
**$p < 0.01$ compared with BBN alone.
*$p < 0.001$ compared with BBN alone.

We examined the promoting effect of saccharin after initiation with two different doses of BBN. Male 6-week-old F344 rats were divided into five groups. In the initiation stage, the rats were given drinking water containing solutions of 0.05%, 0.01% or 0% BBN for 4 weeks. Then in the promotion stage, they were given diet containing 5% saccharin or basal diet for 32 weeks.

The incidences and numbers of PN hyperplasia per 10 cm of basement membrane in the urinary bladder are shown in Table 4. The incidence and number of PN hyperplasia and cancer in the groups that received 0.01 or 0.05% BBN followed by saccharin was significantly higher than in the respective BBN groups without saccharin. In the groups treated with 0.01% BBN, administration of saccharin also increased the occurrence of PN hyperplasia significantly. The inductions of PN hyperplasia and cancer in the group treated with the high dose of BBN followed by saccharin were greater than those in the group treated with the low dose of BBN followed by saccharin. Thus, in the present study, the two different doses of BBN showed a dose-response relationship for initiation of urinary bladder carcinogenesis that was promoted by saccharin. These results are consistent with those on the promoting effect of phenacetin in two-stage carcinogenesis or rat urinary bladder initiated by two different doses of BBN (*9*).

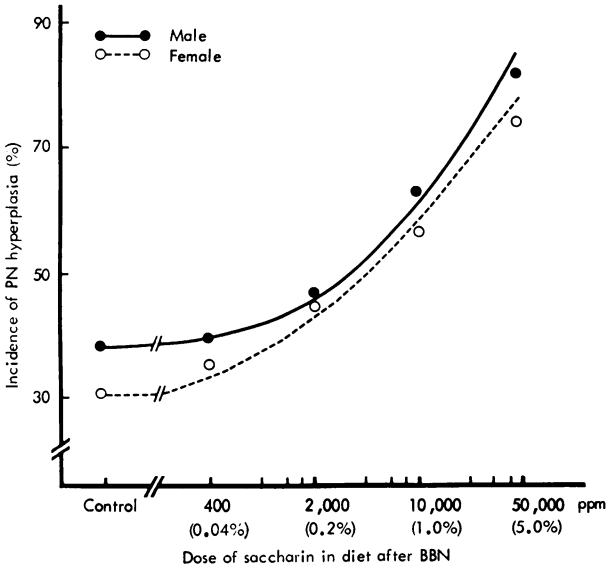

FIGURE 4. Dose-response curve for incidence of papillary or nodular hyperplasia in rat urinary bladder.

Organ-Specific Effects of Promoters

For study of the organ specific effects of two different tumor promoters, rats were divided into eight groups. In the initiation stage, the rats were given 0.02% 2-AAF in the diet for 4 weeks, or 0.01% BBN in the drinking water for 4 weeks, or neither 2-AAF nor BBN for 4 weeks. Then, in the promotion stage, they were given 0.05% phenobarbital in the diet, or 5.0% saccharin in the diet, or basal diet for 32 weeks. The experimental protocol is shown in Figure 5. Phenobarbital greatly en-

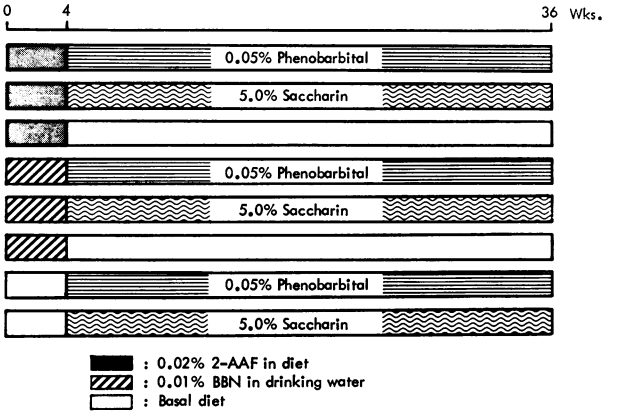

FIGURE 5. Experimental protocol for testing organ specificity of promoters in two-stage carcinogenesis in rat liver or urinary bladder.

Table 4. Lesions of the urinary bladder in male rats initiated by different doses of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) followed by 5% saccharin for 32 weeks.

Dose of BBN, %	Treatment with saccharin	No. of rats	PN hyperplasia[a]		Carcinoma[a]	
			Incidence, %	No./10 cm of basement membrane	Incidence, %	No./10 cm of basement membrane
0.05	+	25	25 (100) *	26.2 ± 18.1*	20 (80.0)*	1.3 ± 1.2*
0.05	–	26	12 (46.2)	0.7 ± 0.9	1 (3.8)	0.0 ± 0.1
0.01	+	29	24 (82.8)**	2.6 ± 2.2**	0 —	0
0.01	–	28	11 (39.3)	0.7 ± 1.0	0 —	0
0	+	28	0 —	0	0 —	0

[a]Values are means ± SD.
*$p < 0.001$ compared with 0.05% BBN alone.
**$p < 0.001$ compared with 0.01% BBN alone.

hanced hepatocarcinogenesis and induced hepatocellular carcinoma after 2-AAF treatment, and it significantly increased formation of hyperplastic (neoplastic) liver nodules after BBN treatment (Table 5). Saccharin significantly enhanced the induction of PN hyperplasias of the urinary bladder after BBN or 2- AAF treatment (Table 6). However, after 2-AAF or BBN treatment, there was no effect of phenobarbital on the urinary bladder or of saccharin on liver neoplasia induction. These data indicate that although 2-AAF and BBN have tumor-initiating effects in both liver and urinary bladder, the tumor- promoting effects of phenobarbital and saccharin are organ specific. The results are consistent with the findings that phenobarbital did not enhance 7,12-dimethylbenz(a)anthracene-induced skin tumors (*16*) or dimethylnitrosamine-induced lung tumors and that phenobarbital increased the incidence of spontaneous liver tumors, but had no effect on the incidence of other spontaneous tumors (*17*). Saccharin also did not enhance induction of forestomach tumors by benzo(a)pyrene (*18*). Therefore, the tumor-promoting effects of phenobarbital and saccharin may be restricted to the liver and urinary bladder, respectively.

Strain Differences in Susceptibility of the Urinary Bladder to Saccharin

There are many reported differences in chemical carcinogenesis between strains of animals (*7,19*). Strain difference is one of the factors that modify chemical carcinogenesis. Strain differences appear to modify promotion as well as initiation in two-stage carcinogenesis. Therefore, we tested whether there was any strain difference in the effect of saccharin as a promoter.

Male 6-week-old ACI, Wistar, F344 and Sprague Dawley (SD) rats were given diet with 5% saccharin for 52 weeks or basal diet for 52 weeks as a con-

Table 5. Lesions of the liver in male rats initiated by 2-acetylaminofluorene (2-AAF) or *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) followed by phenobarbital (PB) or saccharin (S).

		Hyperplastic nodule			Hepatocellular carcinoma		
Treatment[a]	No. of rats	Incidence, %	No./cm²[b]	Area, mm²/cm²[b]	Incidence, %	No./cm²[b]	Area, mm²/cm²[b]
2-AAF⟶PB	24	24 (100)	5.5 ± 3.2*	3.5 ± 2.1*	5 (20.8)*	0.0 ± 0.1**	1.3 ± 3.0*
2-AAF⟶S	29	27 (93.1)	3.0 ± 3.6	1.9 ± 2.4	0 —	0	0
2-AAF	28	27 (96.4)	3.6 ± 2.6	1.9 ± 1.8	0 —	0	0
BBN⟶PB	30	8 (26.6)*	<0.1	<0.1	0 —	0	0
BBN⟶S	29	0 —	0	0	0 —	0	0
BBN	28	0 —	0	0	0 —	0	0
PB	29	0 —	0	0	0 —	0	0
S	28	0 —	0	0	0 —	0	0
—	29	0 —	0	0	0 —	0	0

[a]The rats were initiated with either 0.02% 2-AAF in their diet or 0.01% BBN in their drinking water for 4 weeks followed by either 0.05% phenobarbital or 5.0% saccharin in their diet for 32 weeks.
[b]Values are means ± SD.
*$p < 0.05$ compared with 2-AAF alone.
**$p < 0.01$ compared with BBN alone.

Table 6. Lesions of the urinary bladder in male rats initiated with 2-acetylaminofluorene (2-AAF) or *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) followed by phenobarbital (PB) or saccharin (S).

		PN hyperplasia		Papilloma	
Treatment[a]	No. of rats	Incidence, %	No./10 cm of basement membrane[b]	Incidence, %	No./10 cm of basement membrane[b]
2-AAF⟶PB	24	0 —	0	0 —	0
2-AAF⟶S	29	4 (13.8)*	0.3 ± 1.1	0 —	0
2-AAF	28	0 —	0	0 —	0
BBN⟶PB	30	14 (46.7)	0.6 ± 0.7	9 (30.0)	0.4 ± 0.8
BBN⟶S	29	24 (82.8)*	2.6 ± 2.2	6 (20.7)	0.2 ± 0.4
BBN	28	11 (39.3)	0.7 ± 1.0	8 (28.6)	0.4 ± 0.7
PB	29	0 —	0	0 —	0
S	28	0 —	0	0 —	0
—	29	0 —	0	0 —	0

[a]The rats were initiated with either 0.02% 2-AAF in their diet or 0.01% BBN in their drinking water for 4 weeks followed by either 0.05% phenobarbital or 5.0% saccharin in their diet for 32 weeks.
[b]Values are means ± SD.
*$p < 0.05$ compared with 2-AAF alone.

trol, and then their urinary bladder was examined by light and scanning electron microscopy.

Histological examination showed that saccharin induced urinary bladder lesions in ACI rats, but no changes in Wistar, F344 or SD rats (Table 7). PN hyperplasia and papilloma were increased significantly in the urinary bladder of ACI rats given saccharin. Scanning electron microscopy showed mucosal foci with slightly elevated cells, giving a cobblestone appearance, on the luminal surface of the urinary bladder. The superficial epithelial cells were covered with short, uniform microvilli and with ropy or leafy microridges. In addition, several superficial epithelial cells with pleomorphic microvilli on their luminal surface were found in these foci, as described previously (*20*). These scanning electron microscopic findings were greatest in ACI rats among the groups given saccharin. Slight formation of pleomorphic microvilli and short, uniform microvilli were seen in Wistar and F344 rats treated with saccharin, but were not seen in SD rats. Thus, there was a clear strain difference in rats in susceptibility of the urinary bladder to saccharin. It is explained that the promoting effect of saccharin in the urinary bladder was greatest in ACI rats after initiation with naturally occurring compounds. Strain differences may be one of the important factors in explaining differences of exertion of promoting effects.

Membrane Potentials of Urinary Bladder Epithelium in Rats Treated with Saccharin

Scanning electron microscopic studies have shown morphological alterations, such as pleomorphic microvilli and short, uniform microvilli on the bladder surface of rats treated with BBN (*21*), FANFT (*22*), or saccharin in the early stage of the urinary bladder carcinogenesis (*20*) Kakizoe et al. (*23*) reported that Concanavalin A agglutinated isolated bladder cells of rats treated with BBN or saccharin. These findings suggest that urinary bladder carcinogens or promoters might induce changes in physiological function of the surface membrane of the epithelium.

We investigated the electrophysiological changes in the membrane potential of superficial epithelial cells of the urinary bladder in rats exposed to BBN or saccharin. Male 6-week-old F344 rats were given 0.05% BBN in their drinking water or 5% sodium saccharin in their diet for 8 weeks. Untreated rats were used as controls. After 1, 2, 4 or 8 weeks on test, groups of five rats were killed, their urinary bladders removed, everted, and placed on a flat plastic sheet, they were then placed in a chamber filled with modified Krebs solution (35°C) into which 95% O_2 and 5% CO_2 was bubbled. Membrane potentials of superficial epithelial cells of the bladder were measured with a microelectrode filled with 3*M* KCl, and with an impedance of approximately 20 megohms.

The results are summarized in Table 8. Increases in the membrane potential of superficial epithelial cells of the urinary bladder in rats treated with BBN was observed from as early as week 1 to the end of the 8 weeks experiment ($p<0.001$). Saccharin administration also increased membrane potential, the increase being significant in weeks 2 and 4 ($p<0.05$). However, the membrane potentials induced by saccharin were consistently lower than those induced by BBN. In general, change in membrane potential occur due to a change in K^+ permeability and/or change of Na^- electrogenic pump activity so that the increase of membrane potential in groups treated with BBN and saccharin might reflect these changes. Differences between the membrane potentials of groups treated with BBN and saccharin, therefore, may be related to differences in membrane components.

Table 7. Histological and scanning electron microscopic changes of the urinary bladder in four strains of male rats receiving diets containing 5% saccharin for 52 weeks.

Strain	Treatment with saccharin	No. of rats	Histology: PN hyperplasia, %	Histology: Papilloma, %	Histology: Carcinoma, %	Scanning electron microscopy: Pleomorphic microvilli	Scanning electron microscopy: Short, uniform microvilli
ACI	+	32	20 (62.5)*	9 (28.1)**	3 (9.4)	+ + +	+ + +
	−	28	0 −	0 −	0 −	−	−
Wistar	+	26	0 −	0 −	0 −	+	+
	−	24	0 −	0 −	0 −	−	−
F344	+	25	0 −	0 −	0 −	+	+
	−	25	0 −	0 −	0 −	−	−
Sprague-Dawley	+	26	0 −	0 −	0 −	−	−
	−	26	0 −	0 −	0 −	−	−

**$p < 0.01$ compared with no saccharin.
*$p < 0.001$ compared with no saccharin.

Table 8. Membrane potentials of the epithelium of F344 male rat urinary bladder treated with 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) or 5% saccharin.

Chemical	Membrane potential, -mV[a]			
	At 1 week	At 2 weeks	At 4 weeks	At 8 weeks
BBN	35.3 ± 3.3*	35.8 ± 1.5*	38.0 ± 1.9*	39.0 ± 3.5*
Saccharin	28.9 ± 1.9	31.7 ± 3.1**	31.0 ± 2.5**	33.3 ± 1.6
Control	25.6 ± 2.9	25.3 ± 2.1	25.5 ± 1.3	30.4 ± 2.0

[a]Values are means ± SD of groups consisting of five rats each.
**$p < 0.05$ compared with control.
*$p < 0.001$ compared with control.

Conclusions

The two-stage process, initiation and promotion, applies to urinary bladder carcinogenesis. The promoting effects of 16 test chemicals on urinary bladder carcinogenesis in rats initiated by BBN were examined, and the *in vivo* biological characteristics of the promoter saccharin were examined.

The results showed that various environmental chemicals may have promoting effects on urinary bladder carcinogenesis in rats. The promoting effect of saccharin was dose-dependent and the effects of tumor promoters were organ specific. Moreover, there were strain differences in susceptibility to saccharin.

These findings show that urinary bladder carcinogenesis proceeds by a two-stage process. The concept of promotion is important in assessing the carcinogenic risk to humans of environmental factors. To counteract this risk we must detect not only environmental carcinogens but also environmental promoters. Some short-term *in vitro* tests are recommended for definite evidence of genotoxicity. However, promoters which are epigenetic cannot be detected by short-term *in vitro* test. Therefore, an *in vivo* bioassay using a marker of a preneoplastic lesion, as in the present studies, is useful for detection of promoters of urinary bladder carcinogenesis.

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