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EFFECT OF SODIUM SACCHARIN ON THE NEONATAL RAT BLADDER

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Abstract

In a two-generation bioassay, high doses of dietary sodium saccharin (NaSac) produce bladder carcinoma in rats, whereas acid saccharin (HSac) does not effect the urothelium. NaSac and HSac administered as 5% of the diet to F_0 Sprague-Dawley (SD) and F344 rats, continued through to the weaned male rats for ten additional weeks. Control ^{3}H-thymidine labeling index (LI) was high prior to and at birth (approximately 11%), declining rapidly by weaning (to < 0.2). Neither NaSac nor HSac increased proliferation through 7 days of age. NaSac increased the proliferation rate at later times, whereas HSac did not. The LI decreased to control levels in NaSac-fed rats switched to control diet after weaning and increased in control-fed rats switched to NaSac after birth or weaning. In a second experiment, 5% NaSac did not affect urothelial morphology of SD rats through 7 days. By 21 days post-birth, urothelial hyperplasia occurred in NaSac-fed rat. The LI in treated versus control was similar through gestation, with a slight difference by 7 days. LI was significantly different by 21 days post-birth, but was similar between males and females. These results provide additional evidence for the increased cell proliferative effects of NaSac during the neonatal period, but not during gestation.

Key Words: Bladder, saccharin, proliferation, neonatal development, scanning electron microscopy.

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Introduction

Sodium saccharin (NaSac) produces urinary bladder carcinoma in rats when fed at high doses, but only if administration begins before 35 days of age (Schoenig *et al.*, 1985; Ellwein and Cohen, 1990). Feeding NaSac in the diet beginning with the parental, F_0 male and female rats, continuing in the dams through gestation and lactation and then continuing in the generation F_1 offspring for their two to two and one-half year lifetime causes urinary bladder carcinoma in the F_1 rats (Office of Technology Assessment, 1977; IARC, 1980; Schoenig *et al.*, 1985; Ellwein and Cohen, 1990). A similar response is produced if the NaSac is administered beginning at birth (Schoenig *et al.*, 1985). If administration begins at approximately 35 days of age, bladder cancer occurs at lower incidences (Arnold *et al.*, 1980). Beginning administration at 6-8 weeks of age, the usual practice in the standard two-year bioassay, produced no cases or an insignificant incidence of bladder cancer (Office of Technology Assessment, 1977; IARC, 1980; Schoenig *et al.*, 1985; Ellwein and Cohen, 1990).

During the neonatal time period, the NaSac dose, expressed in terms of mg/kg body weight, increases to a peak level at 30 days of age, when rats are fed a constant dietary level and then decreases over time (Schoenig and Anderson, 1985). Also, the amount consumed on a per weight basis is greater in the female offspring than in the male (Schoenig and Anderson, 1985).

Hyperlipidemia, mild changes in vitamin A and vitamin D levels in the blood and in the liver, and decreases in blood folate levels occur in male and female offspring administered varying doses of NaSac during the neonatal period (Garland *et al.*, 1991a; 1991b; 1993). In addition, the generation F_1 offspring develop anemia during the lactational period, which is predominantly associated with an iron deficiency. The various hematologic, biochemical and nutritional effects appear to be dose-related, with a no-effect level seen at 1% of the diet (Garland *et al.*, 1991b). In addition, changes appearing through 30 days of age reverse toward normal by 90 days, even if the rats continue on the high doses of

NaSac in the diet (Garland *et al.*, 1991b). With respect to the hyperlipidemia, the changes not only reverse, but by 90 days of age, the rats show evidence of a hypolipidemia (Garland *et al.*, 1991b).

Most of these hematological, nutritional and biochemical changes in neonatal rats administered high doses of NaSac can be reversed by supplementing the diet with high doses of iron, administered as carbonyl iron (Garland *et al.*, 1991b). Some of the changes are reversed by folate supplementation, although reversal is not complete for the hematologic and lipid changes. However, instead of inhibiting the urinary bladder proliferative changes, supplementation with iron and/or folate has either no effect or a slight potentiating effect.

Saccharin is a relatively strong acid with a pK_a of approximately 2.8 (Ellwein and Cohen, 1990), which means that it is completely ionized under physiological conditions, including excretion in the urine. It is passed to the offspring through the dams' milk, also in ionized form (Renwick, 1985). It is not metabolized in the rat or other species, including humans (Sweatman and Renwick, 1979). Since it is nucleophilic rather than electrophilic and does not generate metabolites which are electrophilic, it does not have the potential for interacting with DNA (Lutz and Schlatter, 1977). In most short-term assays, saccharin is non-genotoxic (Ashby, 1985; Ashby and Ishidate, 1986).

Administration of high doses of NaSac to adult rats increases urothelial proliferation, as evidenced by the presence of hyperplasia and by an increase in the labeling index, following a pulse dose of tritiated thymidine or bromodeoxyuridine (Ellwein and Cohen, 1990). In contrast, similar or even higher doses of acid saccharin (HSac) do not produce such an effect (Hasegawa and Cohen, 1986; Cohen *et al.*, 1991), probably due to acidification of the urine. Feeding NaSac produces approximately neutral urinary pH. However, if NaSac is co-administered with ammonium chloride (Ellwein and Cohen, 1990; Cohen *et al.*, 1991) or is administered in semisynthetic AIN-76A diet (Ellwein and Cohen, 1990; Okamura *et al.*, 1991), (both circumstances producing a strongly acidic urine with pH below 6.0), the proliferative and tumorigenic effects of the NaSac are inhibited.

During gestation and lactation, the urinary bladder epithelium evolves rapidly through numerous morphologic changes before acquiring the appearance of the adult (Firth and Hicks, 1972; Ayres *et al.*, 1985; Cohen *et al.*, 1988). Several features are present *in utero* and during the neonatal time period which are not normally seen in the adult bladder epithelium, except during carcinogenesis or in carcinomas or in cell culture of urothelium, particularly of malignantly transformed rat urothelium (Cohen *et al.*, 1988). Also, the adult bladder is a mitotically quiescent tissue with few mitoses, whereas, *in utero* the bladder epithelium has numerous mitoses, particularly prior to day 17 of gestation in the rat (Firth and Hicks, 1972; Ayres *et al.*, 1985; Cohen *et al.*, 1988; Ellwein and Cohen, 1990). The following studies using light microscopy, scanning electron microscopy (SEM), and labeling index methods (Cohen *et al.*, 1990) were performed to evaluate whether high doses of saccharin administered to rats affect either the morphologic or proliferative aspects of the bladder epithelium during gestation and lactation compared to after weaning.

Materials and Methods

Materials

Sprague-Dawley rats were purchased from Charles River Laboratories in Portage, MI, and F344 rats were from Charles River Laboratories in Kingston, NY. The rats' diet was Prolab 3200 rat chow (Agway, Inc., St. Marys, OH), and their water was deionized and distilled. NaSac and acid saccharin were provided by Sherwin Williams (Cincinnati, OH), and were mixed in the diet as 5% by weight and pelleted by Dyets, Inc. (Bethlehem, PA). Chemical samples were analyzed by NMR for evaluation of identity and purity. Control animals were fed the pelleted rat chow without added chemicals.

Animal Care and Methods

Male rats were received at 8 (Experiment 1) or 7 (Experiment 2) weeks of age and the females at 6 weeks of age. Upon arrival, the rats were kept in quarantine for two weeks and fed control diet. They were housed 5 per cage in polycarbonate cages with corncob bedding. The animals were kept on a 12 hour light/dark schedule. The temperature was targeted to be 21 $\pm$ 2°C and the humidity at 50 $\pm$ 15%.

After the two week quarantine period, the rats were weighed and randomized (Martin *et al.*, 1984) into the respective experimental groups. At this time, the rats were identified by ear tag, and each group of rats began its respective dietary regimen.

Mating was begun after a two week period of feeding the respective diets. The first day of successful mating was considered day zero of gestation. The F_0 males were sacrificed on completion of the mating period. Females which did not mate were sacrificed at the end of the mating period or as soon as it became apparent that what had been considered a successful mating did not actually result in pregnancy. At the time of birth, the number of males and females in each litter was recorded and the pups weighed. Culling was performed at day 4 after birth, reducing the litter size to 10, with 5 males and 5 females when possible.

Diet and water consumption were measured during weeks 1 and 2 of the diet feeding period before mating.

The F_0 rats were weighed at the time of randomization and again at the end of 1 and 2 weeks of feeding. The F_0 females and their offspring were weighed immediately after birth, days 4, 7, 14 and 21 postnatally, and at the time of sacrifice.

Tissue harvesting, processing and examination

The rats were anesthetized by nembutal injection intraperitoneally (i.p). The fetal bladders were obtained as described previously (Cano *et al.*, 1986; Cohen *et al.*, 1988). Briefly, the fetuses were removed by caesarian section and immediately immersed in glutaraldehyde fixative. For the rats sacrificed during the lactation period, the bladders were exposed through an abdominal incision under nembutal anesthesia. The bladders were inflated *in situ* with glutaraldehyde fixative in phosphate buffer (pH 7.4), ligated, removed and placed in fixative for further processing.

Bladders from rats sacrificed during lactation or after weaning were divided in half sagittally, with one-half processed for SEM in Experiment 2 and the other for light microscopy and autoradiography (Cohen *et al.*, 1990). Both halves were processed for autoradiography and histopathology in Experiment 1. The half to be examined by SEM was processed as described previously (Cohen *et al.*, 1990); the process included critical-point drying, mounting on an aluminum stub, and then incising around the periphery to allow the bladder to be flattened onto the stub. Once on the stub, additional horizontal and longitudinal incisions were made completely across the mounted bladder, resulting in a grid that allowed scanning of one grid section at a time. This method allowed accurate mapping and quantitation of bladder epithelium.

The specimens for light microscopy and autoradiography were embedded in paraffin after dehydration using an ascending series of ethanol concentrations and xylene. Sections were cut 4-5 μm thick, processed for autoradiography, and then stained with hematoxylin and eosin.

Autoradiography

^{3}H-thymidine (New England Nuclear, Boston, MA) (20 Ci/mM) was injected i.p. into the rats at a level of 1 μCi/g body weight one hour before sacrifice. Rats were sacrificed during a two hour time period (1000-1200 hours) to avoid diurnal variations in the labeling index (Tiltman and Friedell, 1972). The animals were weighed just before injection of the thymidine. A piece of forestomach was also evaluated, to serve as a rapidly proliferating, positive control tissue. For sacrifice of the neonatal rats at the early time points (days 2, 4 and 7 after birth), 1 ml of ^{3}H-thymidine as received was diluted with 9 ml of sterile water instead of the usual 4 ml used for the older animals. For obtaining pups while still *in utero*, the mother was injected i.p. using a 1 to 4 dilution. Because of the large size of the rats sacrificed at day 91 in Experiment 1, undiluted tritiated thymidine was used. Processing of paraffin embedded tissues for autoradiography was performed as described previously (Cohen *et al.*, 1990). The unstained slides obtained from paraffin blocks were covered with Kodak nuclear track emulsion, NTB-2, and stored in the refrigerator at 4°C in a light-tight box for 2 weeks. The same batch of emulsion was used for all tissues obtained at a specific sacrifice time point. The slides were developed in Kodak D-19 developer and fixer, then rinsed and stained with hematoxylin and eosin for histopathology and autoradiographic labeling index determinations. Eight grains per nucleus was considered a positively-labeled cell.

Table 1. The number of rats randomly allocated to each group in the F_0 generation for Experiment 1.

Group	Strain[a]	# of Rats	
		Female	Male
1 + 1A	SD	40	20
2	SD	40	20
3+ 3A + 3B	SD	55	28
4	F344	16	8
5	F344	16	8
6	F344	16	8

[a]SD = Sprague Dawley; F344 = Fischer 344

Histopathologic and SEM Evaluation

The slides processed for autoradiography were also evaluated for light microscopic histopathology. This was primarily to observe differences in thickness of the epithelium with respect to cell number. Also, the presence of superficial umbrella cells was determined, and nuclear and cytologic parameters were also evaluated.

The features evaluated by SEM were those previously described as occurring during gestation and lactation (Cohen *et al.*, 1988). This included examination of the bladders for the presence of cilia, octopus-like cells, bridge cells, superficial polygonal cells, microridges, uniform microvilli, pores, and the appearance of a uniform hexagonal array of large superficial umbrella cells with leafy microridges characteristic of the adult bladder. The temporal occurrence of these features during the perinatal time period has been defined in previous studies (Cohen *et al.*, 1988). In addition, examination of the bladders at days 7 and 21 of age, just before weaning, included evaluation for the presence of

Table 2. Treatments and sacrifice schedule for Experiment 1.

Group	Strain[a]	Treatment	Total # of liters	# of rats obtained at various times[b] 19G[c]	21G[c]	2	4	7	14	21	28	63	91
1	SD	5% NaSac	17	10	10	9	10	7	10	11		10	11
1A	SD	5% NaSac during gestation & lactation → 6 wks control diet → 4 wks 5% NaSac	4									7	10
2	SD	5% acid saccharin	20	10	10	8	10	10	10	10		8	9
3	SD	Control	16	10	10	10	10	10	11	10		10	11
3A	SD	Control during gestation → 5% NaSac during lactation → control for 10 wks.	12			8	11		10	10			10
3B	SD	Control during gestation and lactation → 10 wks. 5% NaSac	4									8	9
4	F344	5%NaSac	5		10			10			9		5
5	F344	5% acid saccharin	4		8			6			9		0
6	F344	Control	7		10			8			6		11

[a]SD = Sprague Dawley; F344 = Fischer F344.
[b]Actual number of male rats examined at each time point.
[c]Rats sacrificed at days 19 and 21 days of gestation; day 21 of gestation was the expected day of giving birth.

necrosis and proliferation as previously defined (Cano *et al.*, 1986; Cohen *et al.*, 1988).

Statistical analyses

Statistical comparisons between groups for all of the parameters were made by the generalized linear model (SAS/STAT, 1989). A p value of ≤ 0.05 was considered statistically significant.

Experiment 1

Seventy-one male and 141 female Sprague-Dawley rats and 25 male and 48 female F344 rats were obtained for breeding for Experiment 1. After the 2 week quarantine period, the rats were divided into three groups for each of the strains, with the number of animals in each group shown in Table 1. The excess rats were used for health surveillance examination or discarded. At the time of birth, the various litters in the three original Sprague-Dawley groups were further divided into subgroups, depending on their dietary regimens.

The treatment for each group is given in Table 2 along with the specific schedule for labeling index analysis and histopathology. Weaning occurred at 21 days after birth for the Sprague-Dawley rats and 28 days after birth for the F344 rats. Only male offspring were evaluated in this experiment except for days 19 and 21 of gestation, when the F_1 females were also evaluated. However, the pregnant dams at 21 days of gestation did not adequately absorb the ^{3}H-thymidine from the i.p. injections, eliminating this time point from further analysis. Occasionally, rats at other time points did not absorb the ^{3}H-thymidine, usually as a consequence of injection into greatly enlarged cecums in the NaSac-treated rats. At the time of weaning, the F_0 females and all remaining F_1 females were sacrificed.

Except for routine bladder histopathology evaluation in five F_0 males and five F_0 females for each of the groups, no tissue analysis or autoradiography was done on F_0 rats, males or females. This routine histopathology evaluation was done as a check on the quality of the rats received from the supplier.

At the time of weaning, the offspring from each litter were housed in one cage if there were 5 or fewer males per litter, or divided into two cages if there were 6 or more males in a litter.

Experiment 2

Forty male and 80 female Sprague-Dawley rats were obtained and randomized into two groups of equal size after two weeks in quarantine. Additional males and females were also ordered for health screens, including necropsy and hematology at arrival. Group 1 served as the controls and Group 2 were administered NaSac as 5% of the diet. The mating and offspring sacrifice dates were scheduled so that offspring of a specific age could

Table 3. Body weight of F_0 and F_1 rats at different times in the treatment groups in Experiment 1.

Group		Pups/ Litter	Body weight (Mean ± standard error of mean, S.E.) F_0 - day 0	F_0 - day 14	F_0 Gestation Day 20	Pups - Birth	Pups - Day 21	Pups - Day 28	Pups - Day 91
1	NaSac Female	6.3±0.5[a]	154±1	199±2	325±6[c]	6.2±0.1			
	NaSac Male	5.9±0.5	261±3	345±4[c]		6.6±0.1	44±1		439±16[c]
1A	NaSac → Cont. → NaSac Male						45±1		446±13[c]
2	Acid Sac. Female	6.5±0.5	150±1[c]	187±2[c]	320±5[c]	6.0±0.1			
	Acid Sac. Male	5.8±0.5	257±3[c]	333±5[c]		6.4±0.2	40±1[c]		384±8[c]
3	Control Female	7.1±0.4[b]	158±1	204±2	353±4	6.0±0.1			
	Control Male	6.7±0.3	265±3	362±4		6.3±0.1	48±2		522±11[e]
3A	Cont. → NaSac → Cont. Male						45±1		478±15[c]
3B	Cont. → NaSac Male						50±2[c]		420±9[c]
4	NaSac Female (F344)	5.2±0.4	100±1	131±1	199±7	4.6±0.1			
	NaSac Male	4.0±0.6	168±3[c]	207±4[c]		4.8±0.1		41±1[c]	263±6
5	Acid Sac Female (F344)	5.0±0.8	97±1[c]	124±1[c]	181±11[d]	4.3±0.1			
	Acid Sac Male	3.8±0.6	164±2[c]	199±5[c]		4.6±0.1[c]	31±1[c]		
6	Control Female (F344)	4.7±0.6	102±1	133±1	199±10	4.7±0.1			
	Control Male	5.3±0.4	176±2	217±3		5.1±0.2		54±2	283±11

[a]Includes litters from Groups 1 and 1A before dividing into 2 groups.
[b]Includes litters from Groups 3, 3A and 3B before dividing into 3 groups.
[c]$p < 0.05$ compared to respective control group.
[d]$p < 0.08$ compared to group 6.
[e]Sacrificed at day 94 instead of day 91 or 92.

be sacrificed on the same calendar day for both groups. Autoradiographic, histologic and SEM evaluations were performed on female and male offspring of the F_1 generation, but not on the males and females of the F_0 generation. Histologic evaluation utilized the same slides as those for autoradiography. To the extent possible, 20 male and 20 female offspring (4 litters) per group per time point were to be evaluated. However, to provide sufficient fetal bladder tissue, twice the number of litters per group were sacrificed for days 17 and 21 of gestation, with four of the litters from each group being used for autoradiographic and histologic evaluation and the bladders from four additional litters used for SEM evaluation. A preliminary experiment demonstrated that there was insufficient bladder tissue for both light microscopic and SEM examination of the same bladder. Litters were sacrificed in their entirety. Dating of sacrifice times was from the time of gestation (time from date of mating) for the sacrifice of *in utero* pups, and from the time of birth for subsequent sacrifice times. Days of sacrifice were 17 and 21 of gestation and 7 and 21 of lactation.

Results

Experiment 1

Similar to other experiments (Schoenig *et al.*, 1985; Garland *et al.*, 1991a, 1991b, 1993), NaSac administered to the F_0 generation produced a slight decrease in weight gain during the prefeeding period prior to mating, more in male than female rats and more in the Sprague-Dawley rats than F344 rats (Table 3). Acid saccharin also produced a significant decrease in weight gain, and there was a significantly smaller weight gain than with NaSac. During gestation, the Sprague-Dawley dams administered either NaSac or acid saccharin gained significantly less weight than the controls. At birth, the pups were similar in weight between all groups within each strain, and the number of offspring per litter in each group was similar between groups (Table 3). There was no evidence of malformation. By the time of weaning, however, the acid saccharin-administered offspring in both Sprague-Dawley and F344 rats were significantly lighter than the controls.

Overall, food consumption was decreased in the treated rats compared to the controls, but the water consumption was significantly increased in the rats during

Table 4. Labeling index of the bladder urothelium in rats in the various treatment groups at different times in Experiment 1 treatment groups.

Group	Labeling index at various times (Mean ± S.E.)									
	19G-M*	19G-F*	2	4	7	14	21	28	63	91
1	9.9±1.0	8.0±1.2[a]	7.9±0.8[b]	3.3±0.4[c]	2.0±0.3[b]	1.9±0.4	0.68±0.42[c]		0.25±0.08[a]	0.29±0.10[c]
1A									0.12±0.03	0.13±0.05
2	6.5±0.6	5.3±0.8	7.7±0.8[b]	2.4±0.1	2.4±0.2[b]	4.4±1.0[b]	0.26±0.11		0.13±0.03	0.02±0.01[b,d]
3	6.0±1.3	6.3±1.1	11.1±1.3	1.8±0.3	4.0±0.6	1.5±0.4	0.12±0.05		0.10±0.02	0.09±0.03
3A			5.0±0.8[b]	4.7±1.0[b]		3.2±0.5[c]	0.11±0.04			0.09±0.01
3B									0.17±0.07	0.53±0.18[b,d]
4					1.4±0.2			0.27±0.13[e]		0.36±0.23[f]
5					0.7±0.3			0.07±0.03		
6					1.0±0.3			0.02±0.01		0.10±0.04

*Day 19 of gestation, males and females, respectively. Remainder of times refer to days after birth.
[a]$p < 0.06$ Compared to Group 3; [b]$p < 0.05$ Compared to Group 3; [c]$p < 0.1$ Compared to Group 3;
[d]$p < 0.05$ Compared to Group 1; [e]$p < 0.08$ Compared to Group 6; [f]$p < 0.12$ Compared to Group 6.

Table 5. Body weight of F_0 and F_1 rats at different times in Experiment 2.

Group	Body weight (Mean ± S.E.)								
	F_0 - day 0	F_0 - day 14	F_0 Gestation - Day 16	F_0 Gestation - Day 21	F_0 Dams Lactation - Day 4	F_0 Dams Lactation - Day 21	Pups - birth	Pups - Day 7	Pups - Day 21
Control Female	190±1	225±2	299±3	363±4	291±4	306±7	6.3±0.1	15.3±0.7	50±3
NaSac Female	190±1	218±2[a]	291±4	356±5	293±5	332±4[a]	6.4±0.2	14.3±0.4	41±1[a]
Control Male	315±3	410±5[a]					6.6±0.2	15.8±0.8	52±3
NaSac Male	313±2	372±4					6.8±0.2	14.8±0.4	42±1[a]

[a]Significantly different than controls, $p < 0.05$.

Table 6. Litter size for rats in Experiment 2.

	Avg. no. of pups per litter
Control female	6.5 ± 0.8
Control male	6.1 ± 0.7
Control total	12.6 ± 0.8
NaSac female	6.5 ± 0.7
NaSac male	5.3 ± 0.5
NaSac Total	11.7 ± 0.7

NaSac treatment (data not shown) as observed previously (Schoenig *et al.*, 1985; Garland *et al.*, 1991a, 1991b, 1993). Increased water consumption was not observed with acid saccharin.

Histopathologic examination revealed no abnormalities in the bladders through the time of weaning. However, by day 91 of the experiment, rats continuing treatment with NaSac (Groups 1, 3B, and 4) showed mild simple hyperplasia.

The results of the labeling index evaluations are presented in Table 4. The labeling index prior to and just after birth was high, ranging from 5.0 to 11.1% in

Table 7. Labeling index of bladder urothelium in F_1 generation control and sodium saccharin-treated rats at different times during gestation and lactation in Experiment 2.

Day	Group	No. of rats	Labeling index (% ± S.E.)
Gestation 17	Control female	36	11.2 ± 0.9
	NaSac[a] female	38	11.9 ± 1.0
	Control male	39	12.6 ± 0.8
	NaSac male	35	12.1 ± 1.0
Gestation 21	Control female	27	8.4 ± 0.9
	NaSac female	41	8.2 ± 0.7
	Control male	37	8.9 ± 0.6
	NaSac female	29	8.4 ± 0.7
Post-Birth 7	Control female	18	1.3 ± 0.2
	NaSac female	19	1.8 ± 0.3
	Control male	22	1.5 ± 0.2
	NaSac male	19	1.7 ± 0.3
Post-Birth 21	Control female	19	0.26 ± 0.06
	NaSac female	19	0.44 ± 0.12[b]
	Control male	21	0.16 ± 0.03
	NaSac male	21	0.36 ± 0.05[c]

[a]NaSac = 5% sodium saccharin in the diet. [b]$p < 0.07$ for NaSac-treated females compared to control females. [c]$p < 0.05$ for NaSac-treated males compared to control males.

the various groups. Although the rate was elevated two days prior to birth in the NaSac treated group compared to controls ($p < 0.06$), by two days after birth, the acid saccharin and NaSac-treated rats actually had a labeling index that was less than controls. There seemed to be considerable fluctuation above and below control levels through day 7 of administration, and probably does not represent a true difference between groups.

At day 14, the rats treated with acid saccharin had a significantly higher labeling index than either the controls or the NaSac-treated group. Otherwise, rats treated with acid saccharin were similar to controls. The F344 rats administered acid saccharin did not produce sufficient litters to be able to have an evaluation at 91 days post-birth. This was due to an insufficient estimate of successful matings to be expected based on normal variation involving a small number of rats. NaSac produced increases in labeling indices in both strains at weaning and at later times, although the statistical significance was often between 0.05 to 0.10 because of the large standard deviations.

Experiment 2

Similar to Experiment 1, NaSac administered to the F_0 generation produced a significant decrease in weight gain in the males but not in the females during the prefeeding period prior to mating (Table 5). During gestation, there was no significant difference between the control dams and the NaSac-treated dams. During lactation, the NaSac treated dams actually gained weight at a slightly greater rate than the controls, becoming statistically significant ($p < 0.01$) by 21 days post-birth. The NaSac-treated F_1 generation rats grew at a slightly slower rate than the controls, becoming statistically significant at day 21 post-birth. The size of the litters in the treated groups compared to controls were similar (Table 6), and there was no evidence of malformations in either group. Overall food consumption was decreased in the treated rats compared to the controls, but the water consumption was significantly increased in the NaSac-treated rats (data not shown). Histopathologic examination revealed no abnormalities in the bladders of any of the treated or control rats at any of the time points.

The results of the labeling index evaluations are presented in Table 7. The labeling index was high during gestation (both days 17 and 21), but rapidly declined by day 7 after birth. These numbers should be compared with those in the usual adult control rat (> 6 wks/age) in which the labeling index is consistently below 0.1% and is frequently below 0.05% (Hasegawa and Cohen, 1986; Cohen *et al.*, 1990; Ellwein and Cohen, 1990). The labeling indices at the different time points were similar between males and females within both control

Table 8. Classification of urothelium of rats in Experiment 2 by scanning electron microscopy[a].

Group	Day	SEM Classification[b]				
		1	2	3	4	5
Female Control	7 post-birth	17	1	0	0	0
Female NaSac[c]	7 post-birth	19	0	0	0	0
Male Control	7 post-birth	16	0	0	0	0
Male NaSac	7 post-birth	11	0	0	0	0
Female Control	21 post-birth	16	3	0	0	0
Female NaSac	21 post-birth	2	1	3	3	0
Male Control	21 post-birth	16	5	0	0	0
Male NaSac	21 post-birth	6	3	0	1	0

[a]All bladders examined at day 17 and 21 of gestation were normal.
[b]Classification according to Cohen *et al.* (1990). Each bladder is tabulated according to its overall appearance.
[c]NaSac = 5% sodium saccharin in the diet.

and treated groups. Differences between treated and control groups did not begin until 7 days post-birth, but became significant by only 21 days of age.

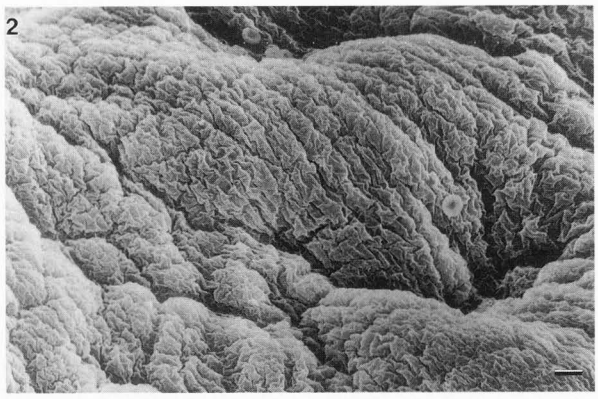
By SEM, the developmental morphologic features of the bladder were the same in male and female rats and in treated compared to control rats. There were no morphologic differences between the treated and control rats during gestation (Fig. 1) or at 7 days post-birth (Fig. 2). By 21 days post-birth (Figs. 3 and 4), however, there was evidence of small foci of superficial necrosis and mild hyperplastic proliferation (Table 8). This was slightly greater in the females than in the males at 21 days post-birth. Occasionally, small amounts of amorphous precipitate was present adherent to the bladder luminal surface (Fig. 5). It was composed predominantly of calcium phosphate as detected by energy dispersive X-ray spectroscopy.

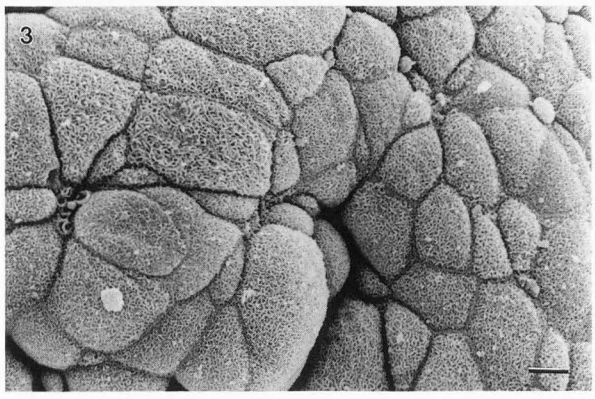
Discussion

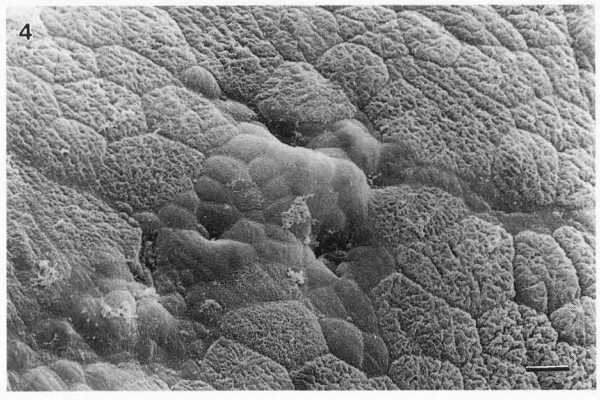
The present studies confirmed previous findings of rapid changes in the developing rat urinary bladder (Firth and Hicks, 1972; Ayres *et al.*, 1985; Cohen *et al.*, 1988), with the urothelium achieving its adult appearance soon after birth. The large superficial polygonal cells are present by day 17 of gestational age, and the various features characteristic of neonatal development have completely disappeared by 21 days of age. The labeling index data confirm the morphologic appearance of a rapidly proliferating tissue *in utero*, with a one-hour pulse labeling in the basal cells of approximately 10% *in utero* and at birth. However, the labeling index declines soon after birth, so that by 21 days of age it is below 0.1%, typical of the adult rat urothelium. This represents a change in proliferation rate of more than two orders of magnitude within the first three weeks of age postnatally.

These studies also demonstrate that high doses of saccharin, whether the sodium salt or the acid form, do not affect the bladder *in utero*, either in terms of effects on morphologic development or on the labeling index. This is to be expected since saccharin does not directly damage DNA, but acts by altering cell proliferation in the urothelium (Lutz and Schlatter, 1977; Ellwein and Cohen, 1990). Since the urothelium during gestation is proliferating essentially at its maximal rate, saccharin cannot enhance the proliferation rate further. It is also important to note that the morphologic development of the bladder progresses normally even with the administration of high doses of either NaSac or acid saccharin.

At seven days of age, the morphology is essentially normal in the saccharin-treated rats, but there is a slight increase in the proliferation rate compared to the control bladder epithelium, although not statistically significant. By day 21 postnatally, the bladder epithelium of the treated male and female rat has a labeling index greater than the control urothelium, and subtle changes of hyperplasia can be detected, especially by SEM. In previous studies in our laboratory, mild hyperplasia was present at 21 days of age and was more pronounced at 30 and 90 days of age with continuous NaSac administration (Garland *et al.*, 1991a, 1991b, 1993). This supports our hypothesis that the effect of NaSac on the neonatal bladder after birth is to increase the proliferation rate, albeit not by a large extent (Ellwein and Cohen, 1988, 1990).







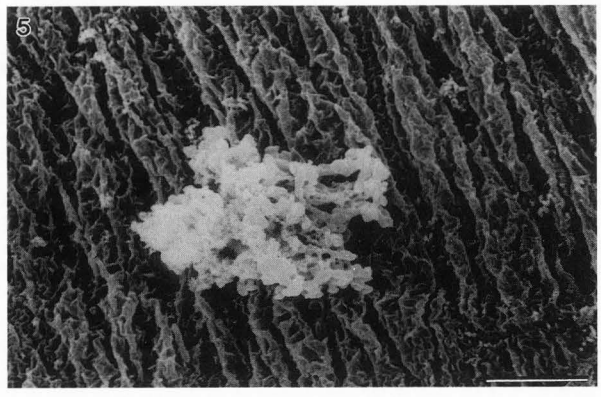


Figure 1. Bladder surface from a male rat at day 21 of gestation in a dam fed diet with 5% sodium saccharin. The appearance is the same as in the controls at this age.

Figure 2. Bladder surface from a male rat at 7 days of age in the group fed sodium saccharin as 5% of the diet. The appearance is the same as in the controls at this age.

Figure 3. Bladder surface from a male rat fed sodium saccharin as 5% of the diet at 21 days of age, showing pleomorphism, rounding of cells with some piling up of cells, and ropy microridges instead of the leafy microridges of normal urothelium. These are the features of mild simple hyperplasia of the urothelium.

Figure 4. Bladder surface from a male rat at 21 days of age, fed sodium saccharin as 5% of the diet, showing areas of necrosis of the superficial cells revealing underlying rounded intermediate cells where there has been exfoliation.

Figure 5. Amorphous precipitate adherent to the surface of the urothelium in a male rat at 21 days of age fed sodium saccharin as 5% of the diet. The major elements present by energy dispersive X-ray spectroscopy were calcium and phosphate.

Bars = 10 μm (Figs. 1-4); and 5 μm (Fig. 5).

Although the increase in proliferation rate is not striking, it occurs in an already rapidly proliferating bladder epithelium that is present early in the life-span of the rat. This is of great biological importance. The adult bladder epithelium is a slowly proliferating tissue in contrast to the epithelium *in utero* and during the first three weeks of life. As a result, it can be calculated that approximately one-third of the total lifetime cell divisions of the urothelium occur during the first three weeks of life (Ellwein and Cohen, 1988). Thus, an increase in proliferation rate and a slight increase in cell number (hyperplasia) during this chronologically short time period represents an enormous increase in the total

number of cell divisions in the lifetime of the rat. This increase in cell proliferation is of disproportionate biological significance because it occurs at the outset of the rat's life. A full two-year lifetime remains for continuous elevation of mitotic rates so long as high doses of NaSac continue to be administered. We have previously (Ellwein and Cohen, 1988) modeled these cellular dynamics, and assuming background levels of genetic errors during DNA replication, the number of cell divisions occurring during this neonatal time period plus those during continued administration for the remainder of the life-span of the rat are adequate to explain the increased incidence of bladder cancer following administration of NaSac beginning at birth. If administration begins at 6-8 weeks of age, the large increase in the number of cell divisions occurring in the early neonatal period of life is lost, with the result that the increased number of cell divisions in the post-weaning period is insufficient to increase the incidence of bladder cancer more than 1%.

In contrast to NaSac, there was essentially no response in the rat urothelium to administration of acid saccharin. This is likely due to the resulting acidification of the urine (Hasegawa and Cohen, 1986; Fisher *et al.*, 1989; Garland *et al.*, 1989; Cohen *et al.*, 1991; Okamura *et al.*, 1991). It is unclear in the present experiment whether the increase at day 14 with acid saccharin in Sprague-Dawley rats has relevance. No differences were observed in F344 rats. The increase at day 14 is a single change and may merely reflect the wide fluctuations in labeling index seen at this time of life.

Somewhat surprisingly in this experiment was the finding that there was little difference in the response to high doses of NaSac in the female and male offspring. Again, with further analysis, this can be readily explained. In previous studies, we have not observed large differences in the urine of the male and female offspring (Experiment 2) of rats administered high doses of NaSac through 30 days of age (Garland *et al.*, 1991a, 1991b, 1993; Cohen *et al.*, 1995). The female appears to receive a higher dose and have a higher urinary concentration of saccharin on a per weight basis (Schoenig and Anderson, 1985; Garland *et al.*, 1991a, 1991b). We have hypothesized that the proliferation that occurs in the rat bladder epithelium is due to the formation of an amorphous precipitate in the urine (Ellwein and Cohen, 1990; Cano *et al.*, 1993; Cohen *et al.*, 1995). It appears that the formation of this precipitate is dependent to a large extent on the presence of high concentrations of protein in the urine along with a variety of other factors that have not yet been defined. During the neonatal time period and continuing to approximately six weeks of age, urinary protein levels in male and female rats are similar and are composed predominantly of albumin. It is not until the sharp increase in urinary α_{2U}-globulin in the male rat beginning at approximately 6 weeks of age that the protein level in the male becomes considerably greater than in the female rat (Hard *et al.*, 1993). After six weeks of age, the effects of NaSac begin to be more significant in the male rat urothelium than in the female (Ellwein and Cohen, 1990; and Cohen *et al.*, unpublished observations). Based on these findings and this analysis, it is not unreasonable to expect male and female rats fed NaSac to have similar morphologic and proliferative changes at 21 days after birth. We have been unable to collect sufficient urine during these first 21 days of age to analyze for the presence of this amorphous precipitate, but precipitate is present on the surface of the bladder urothelium at these early times, including at 7 and 21 days of age post-birth (see Fig. 4).

In summary, the findings in these experiments support our hypothesis that there is an increase in proliferation rate in the bladder epithelium beginning after birth in rats fed high doses of NaSac, and it is similar in the male and female rat through the first 21 days of age. It is the increased proliferation at this early time period, combined with the continuing increased proliferation during the subsequent two years of life of the F_1 generation, that explains the carcinogenic reaction to NaSac. The differential increase in urinary proteins beginning at 6 weeks of age in the male rat helps to explain the greater effects in the male versus the female rat.

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Discussion with Reviewers

C. Frith: The SEM classification scheme should be explained.

Authors: The scanning electron microscopy classification system that we use can be summarized briefly as follows: Class 1 is for the normal bladder, with flat, large, superficial cells. Class 2 is similar, but has an increased number of necrotic and exfoliating cells. Class 3 includes clusters of necrotic cells, the foci 2 to 4 cells in diameter. Also, there is considerable pleomorphism of the superficial polygonal cells. Class 4 includes extensive areas of necrosis revealing numerous small round cells and considerable pleomorphism of the superficial cells. Class 5 involves obvious piling up of small, round cells which have uniform microvilli and occasionally pleomorphic microvilli. For greater detail, please see Cohen *et al.* (1990).

J.B. Reitan: You had problems with ^{3}H-thymidine absorption in pregnant dams, and partly due to enlarged cecums in sodium saccharin-treated rats. Did not the animals absorb ^{3}H-thymidine at all, or was the absorption only reduced? Can such problems represent a significant source of error also in other treatment/age groups?

Authors: If intended intraperitoneal injections end up in the enlarged cecum or in the placental sac, no ^{3}H-thymidine is absorbed. The cecal problem in sodium saccharin-treated rats can occur at any age. It is for these problems that we include a piece of stomach from the injected rat (or pups) to serve as an indicator that a proper injection occurred. The stomach, in contrast to the urothelium, is a rapidly proliferating tissue and will have labelled cells if any ^{3}H-thymidine is absorbed. If the stomach is negative (no labelled cells), a misinjection has occurred.