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COMPARATIVE ANALYSIS OF THE PROLIFERATIVE RESPONSE OF THE RAT URINARY BLADDER TO SODIUM SACCHARIN BY LIGHT AND SCANNING ELECTRON MICROSCOPY AND AUTORADIOGRAPHY

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Abstract

Three methods used to detect proliferative changes in the rat urothelium, light microscopy, scanning electron microscopy, and autoradiography, were compared for their sensitivity in detecting changes produced by administration of sodium saccharin. Weanling male F344 rats were fed sodium saccharin as 0, 3, 5, or 7.5% of the diet, and the bladders were evaluated after 4, 7, and 10 wks of feeding. Light microscopic changes and an increase in labeling index were seen at all time points in rats fed 7.5% sodium saccharin, but not at the lower doses. A slight increase in labeling index was also observed at 10 wks in the 5.0% group. Scanning electron microscopic changes were evident as early as 4 wks with increasing severity at the 3, 5, and 7.5% doses. This study demonstrates that the hyperplastic response of the urothelium to sodium saccharin administration varies with dose and time, and that observation by scanning electron microscopy is the most sensitive of the three methods evaluated for detecting these changes.

Key Words: Scanning Electron Microscopy, Rat Bladder, Epithelium, Saccharin, Hyperplasia, Autoradiography

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Introduction

Sodium saccharin administered at high levels in the diet has been shown to produce urinary bladder tumors in male rats in 2-generation bioassays, and it has also been shown to have co-carcinogenic and promoting activity [6]. Unlike classical carcinogens, it is not metabolized to a reactive electrophile, does not react with DNA, and is not mutagenic. However, a mild increase in proliferation of the urothelium has been observed within a week of administering high doses of sodium saccharin. This proliferative response reaches a maximum within a short period of time and by itself does not appear to lead to the production of urinary bladder tumors if administration begins after weaning [3, 6, 9, 12]. The hyperplasia tends to be mild and multi-focal, and has been detected by light microscopy, scanning electron microscopy, and autoradiography [9, 12]. To further investigate the mechanism by which dietary administration of sodium saccharin leads to increased cell proliferation and possibly tumors in the urinary bladder, it was deemed useful to have a sensitive, reproducible and quantifiable short-term bioassay to evaluate cell proliferation in the urinary bladder epithelium. The purpose of this study was to evaluate the proliferative effect, over time, of various dietary doses of sodium saccharin by comparing the response seen by light microscopy, scanning electron microscopy, and autoradiography.

Materials and Methods

Weanling, 28-day-old, F344 male rats (Charles River Breeding Laboratories, Inc., Kingston, NY) were fed Prolab 3200 diet (Agway, Inc., St.Mary's, OH) pelleted by Dyets, Inc. (Bethlehem, PA) during a 1-wk quarantine period. All rats were housed in polycarbonate cages (16"x18"x20" or 40x45x50 cm) with stainless steel wire bar covers (Lab Products, Inc., Maywood, NJ), 5 rats/cage, on dry corncob bedding at 24±2°C and 50 ± 20% humidity on a 12-hr light/dark cycle. Following the quarantine period, the rats were randomly divided into 12 groups of 10 rats each using the weight stratified method [11]. They were randomly assigned to one of the treatment groups and sacrifice intervals as presented in Table 1.

Sodium saccharin (Sigma Chemical Co., St. Louis, MO) was added to the basal diets on a weight/weight basis. The diet was mixed and pelleted by Dyets, Inc. Food and distilled water were

Table 1: Experimental Design

Dose of Sodium Saccharin (%)	Number of Rats: Sacrifice Interval		
	4 weeks	7 weeks	10 weeks
0(Control)	10	10	10
3.0	10	10	10
5.0	10	10	10
7.5	10	10	10

available ad libitum. Water consumption and diet consumption were determined weekly, over 2-day and 5-day intervals, respectively. Body weights were determined on the days ending the consumption intervals.

Diets were analyzed for saccharin concentration using a modification of the high performance liquid chromatography (HPLC) procedure of Tan and Pan [17]. Diet samples (approximately 0.5 gm) were dried overnight, hydrated with 0.5 ml of 0.1 M NaOH, extracted 3 times with 2.5 ml of 0.1 M NaOH, and centrifuged for 10 min, 1000-2000 rpm (426-852 X g) (Beckman Instruments, Inc., Clinical Instruments Div., Brea, CA) between each extraction. The supernatants were combined in a 10-ml volumetric flask and diluted to volume with triple distilled water. One ml of a 0.122 M stock solution of sodium saccharin was used as an extracted standard. An aliquot (0.5 ml) of the diet extract or standard was applied to a 3-ml disposable (Baker Bond) Quaternary Amine (SAX) column that had been conditioned by sequential washes with methanol, ammonium hydroxide (sp. gr. 0.9), water, and 0.2 M HCl. After application of the sample, the column was washed with two 1-ml aliquots of methanol followed by one 2-ml aliquot of water. The saccharin was eluted into a 5-ml volumetric flask with 2-ml aliquots of 0.2 M K_2HPO_4 buffer (pH 8.8), then diluted to volume with the same buffer. HPLC was performed on a Waters system consisting of a model 510 pump, U6K injector (250 l loop), temperature control module oven, and a model 481 spectrophotometer interfaced with a Hewlett Packard 3390 A integrator. An aliquot (100 µl) of the sample was analyzed using a Waters Novapak C18 column (15 cm x 0.39 cm internal diameter) eluted with methanol:acetic acid:water (166:4:830, pH 3.5) at a flow rate of 1 ml/min at 30°C with ultraviolet (UV) detection at 254 nm, 0.5 absorbance units full scale (AUFS). Ten rats from each group were sacrificed at weeks 4, 7, and 10. A single intraperitoneal injection of [^{3}H-methyl]thymidine (New England Nuclear Co., Boston, MA) was administered to each rat at a dose of 1µCi/g body weight 1 hr before sacrifice. To avoid circadian rhythm-associated variations in labeling index [18] and to control time and handling biases, injections were performed beginning at 9 AM, proceeding with 1 rat from each group, then repeating until all 40 rats were processed.

Under Nembutal anesthesia, urinary bladders were inflated in situ with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The left half of each bladder was marked on the serosal surface with hematoxylin, and the bladder was then removed and placed in the same fixative. Stomachs were inflated in situ with 10% buffered formalin, then removed and placed in formalin. At the 10 wk sacrifice, ceca were removed and weighed immediately. They were then opened, rinsed in saline (9 g NaCl/1000 ml water), blotted gently and weighed again. Code numbers were assigned to each specimen so that they could be evaluated without knowledge of the group to which they belonged. Individual bladders were divided longitudinally in half: The right half was serially cut into 4 longitudinal strips for processing for light microscopy and autoradiography, and the left half was processed for scanning electron microscopy (SEM). A piece of stomach from the junction between glandular and forestomach was included in the processing of the bladder for light microscopy and autoradiography. Urinary bladder and stomach slices for light microscopy were dehydrated and embedded in paraffin. Slides were dipped in Kodak NTB-2 photographic emulsion (1:1 dilution), stored in light-proof boxes at 4°C for 3 weeks, developed with Kodak D-19, and stained with hematoxylin and eosin. For the autoradiographic evaluation, the slides were first reviewed for the presence of grains in the stomach. Grains visible in cells in the forestomach (a rapidly proliferating tissue) indicated that the autoradiography procedure was successful and the bladder could be evaluated. The positive control was necessary since the bladder is a mitotically quiescent tissue, and some bladder specimens were expected to have no labeled cells. All bladder epithelial cells were counted from all strips in one set of sections. When less than 3000 cells were present in one set of sections, an additional serial set of sections of all strips was counted.

The left halves of the bladders were post-fixed in 1% OsO_4 in phosphate buffer, and rapidly dehydrated through an ascending alcohol series. The tissues were critical point-dried (Bomar SPC 1500, Bomar Co., Tacoma, WA) with Freon 13. Specimens were mounted on aluminum specimen stubs using double-sided tape, cut into pieces in a grid-like network (Fig. 1), sputter-coated with gold (Polaron E5100, Hatfield, PA), and examined at 20 kV in an ETEC Autoscan SEM (Perkin-Elmer, Haywood, CA). During examination by scanning electron microscopy, both regular and reverse polarity were used. The entire bladder surface was first evaluated under regular polarity at low magnification, 20-50X, and then again with reverse polarity. The reverse polarity tends to accentuate features such as uniform and pleomorphic microvilli and single cells which are raised slightly above the surface of the remainder of the bladder (Fig. 2). A general overall impression was made of the bladder, and any hyperplastic nodules, folds, or tumors identified. Each of the pieces of the grid were then examined in a back and forth screening fashion at a magnification of approximately 500X. Any distinct features which required further evaluation were examined at a magnification of 1000-2000X. Higher magnifications were occasionally necessary, but rarely greater than 5000X. This process was repeated for each piece on the grid until all pieces were examined.

Normal superficial urothelial cells are covered with leafy microridges with occasional uniform microvilli at the junction between cells. The cells tend to be relatively uniform in size and shape. The features identified were ropy microridges, uniform

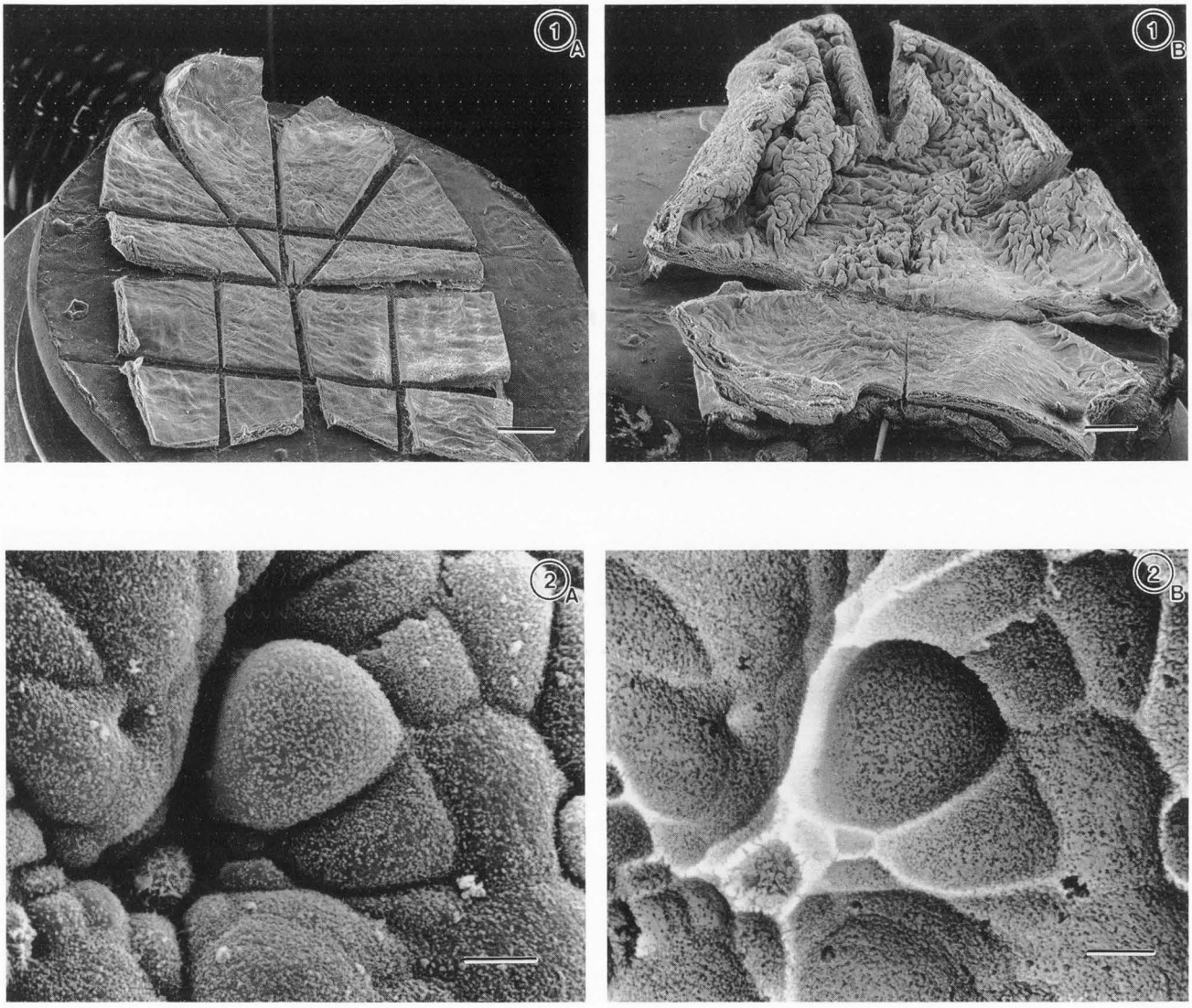


Figure 1. Low magnification views of **(A)** half of a control rat urinary bladder mounted on a specimen stub illustrating the cuts made to allow the bladder to lie flat. It has been divided further into smaller units for screening; and **(B)** a hyperplastic bladder from a rat fed 7.5% sodium saccharin for 10 weeks. (Bars = 1 mm).

Figure 2. (A). Hyperplastic urinary bladder from a rat fed 7.5% sodium saccharin for 10 weeks. **(B).** Reverse polarity of the same area as in (A). (Bars = 2.5 μm).

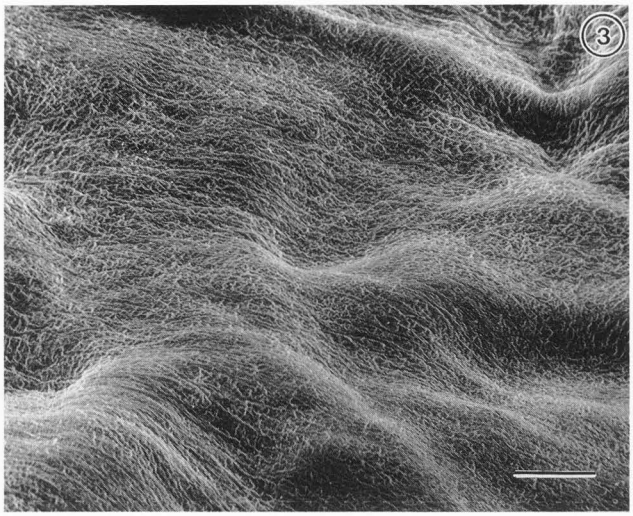
microvilli, and pleomorphic microvilli on individual cells, and pleomorphism of cells, cell necrosis, and piling up of cells. Ropy microridges, uniform microvilli, and pleomorphic microvilli were assessed as defined previously [10]. These features have been identified as being related to increased urothelial proliferation and/or a lack of complete differentiation [9, 10, 12]. Foci were defined as one or more cells which were different from the normal, flat polygonal superficial cells of the rat bladder in either size, shape, or type of feature on the surface. The foci in control bladders represent the normal exfoliation of superficial cells that requires replacement. Based on these features, criteria were defined for assigning a specimen to specific, semi-quantitative classes:

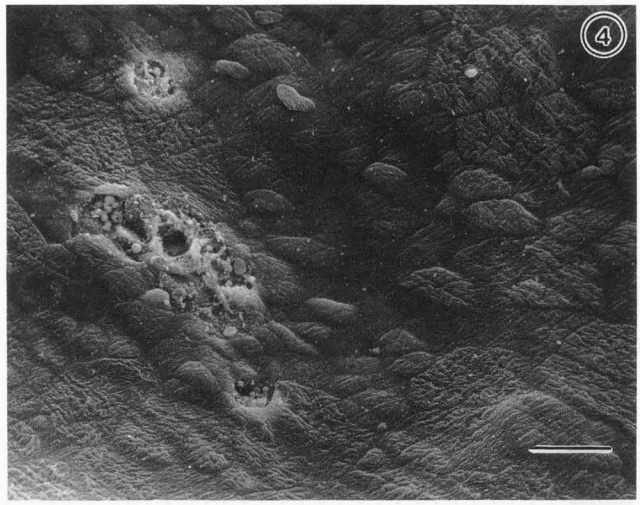
Class I

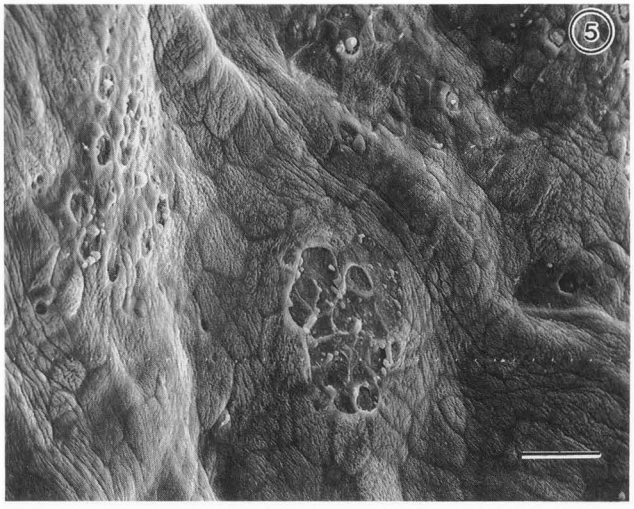
The bladder epithelium is flat and predominantly composed of large polygonal superficial cells with well-developed leafy microridges. Normal cell death and replacement may be present, but there is nearly complete maturation of underlying cells (Fig. 3).

Class II

The bladder epithelium is composed of large, polygonal superficial cells, but there are an increased number of necrotic cells and foci where cells have exfoliated. These are now present in clusters rather than single or paired foci. Some of these areas of necrotic and/or exfoliated cells may have raised cell borders, and the underlying cells that are visualized as the superficial cells exfoliate are not mature. Finally the dome of the bladder may have some







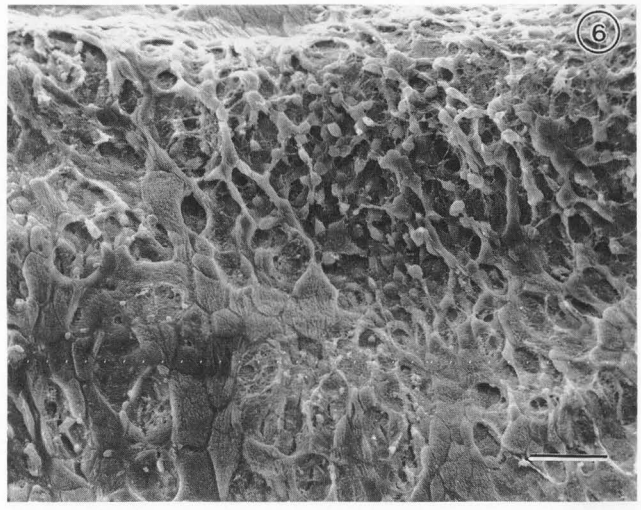


Figure 3. Scanning electron micrograph of a normal urinary bladder (Class I). Note the large polygonal superficial cells with well-developed leafy microridges.

Figure 4. Urinary bladder surface showing polygonal superficial cells with raised cell borders and clusters of exfoliated cells (Class II).

Figure 5. Necrosis and exfoliation of cells throughout the bladder along with pleomorphism of the polygonal cells (Class III).

Figure 6. Extensive necrosis, exfoliation and pleomorphism of cells in the dome of the bladder (Class IV).

Figure 7. Piling up of small, round cells characteristically seen in bladders of (Class V). The surface of the cells is covered with microvilli and ropy microridges rather than the leafy microridges of normal superficial cells.

Bars = 50 micrometers on each figure.

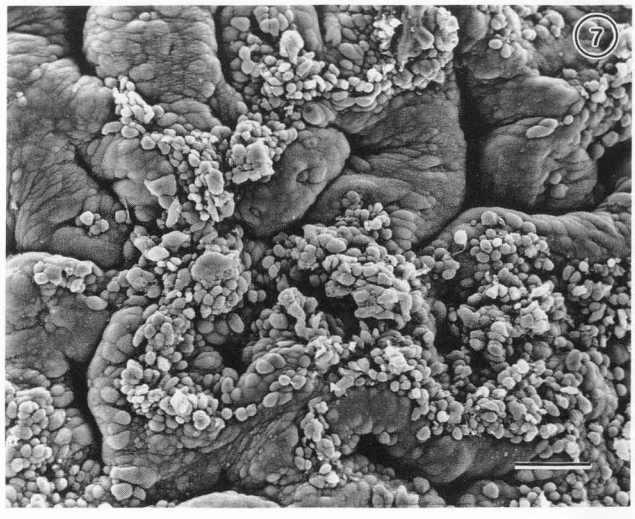


Table 2: Body and Cecal Weights, and Food and Water Consumption, in Rats Fed Various Doses of Sodium Saccharin (NaS)

Group	Level of NaS (%)	Body weight (g)[a] 4 wk.	7 wk.	10 wk.	Water Consumption at 10 weeks (g/rat/day)	Food Consumptiom (g/rat/day) 2wks[b]	10 wks	NaS Consumption (g/rat/ 10 wks)	Cecal Weight at 10 weeks[a] Full (g)	Empty (g)
1	0	226+2	277+3	305+2	24.2	18.9	18.9	0	5.98+0.28	1.28+0.06
2	3	223+2	267+2	302+3	27.8[c]	19.9	19.2	39	9.95+0.25[c]	1.66+0.05[c]
3	5	223+2	260+3	302+5	33.5[c]	23.3[c]	20.7	73	11.78+0.33[c]	1.96+0.09[c]
4	7.5	212+2[d]	257+3[e]	292+3	40.8[c]	28.0[c]	20.7	118	17.08+0.72[c]	2.32+0.10[c]

[a]Mean ± standard error. [b]Week of maximum differences in food consumption between groups.

[c]The value for each successive dose was significantly greater than for preceding level, p less than 0.05.

[d]Significantly lower than Groups 1, 2, 3, p less than 0.05.

[e]Significantly lower than Groups 1 and 2, p less than 0.05.

thickening and folding of the epithelium (Fig. 4).

Class III

The bladder epithelium has considerable pleomorphism of the superficial polygonal cells. Necrosis and areas of exfoliation are present throughout the bladder, comprised of clusters of cells ranging from 2 to 4 cells in diameter. It is usually most severe in the dome portion of the bladder where thickening and folding of the epithelium is also present (Fig. 5).

Class IV

Most of the large, flat, polygonal, superficial cells have exfoliated revealing smaller polygonal cells and round cells as well as cellular debris. The areas of necrosis and exfoliation are extensive and always involve at least 1/2 to 3/4 of the bladder surface. Again there is considerable pleomorphism of cells and the dome of the bladder shows folding (Fig. 6).

Class V

There is extensive necrosis and exfoliation involving most of the bladder surface. Again, the dome of the bladder is the most severely affected, and also exhibits piling up of small, round cells which have uniform microvilli and occasionally pleomorphic microvilli (Fig. 7). Statistical analyses of food and water consumption, body and cecal weights, and autoradiography data were performed using a Generalized Linear Model (GLM) procedure from the Statistical Analysis System (SAS) software package (SAS Institute, Inc., Cary, NC) (SAS User's Guide, 1985). Duncan's multiple-range test [7] and least square means procedure were used for multiple comparison of means.

Results

General Observations

The body weights of the rats fed sodium saccharin generally were lower than those of the control rats, particularly during the first 7 weeks of the study (Table 2). Food consumption was increased in rats fed 5.0% or 7.5% sodium saccharin during the first 4 weeks, but was similar to the control group thereafter. This increased food consumption with the higher doses of sodium saccharin has been noted previously and has been suggested to represent a compensatory increase for the non-nutritive substance in the diet [16]. Daily food consumption

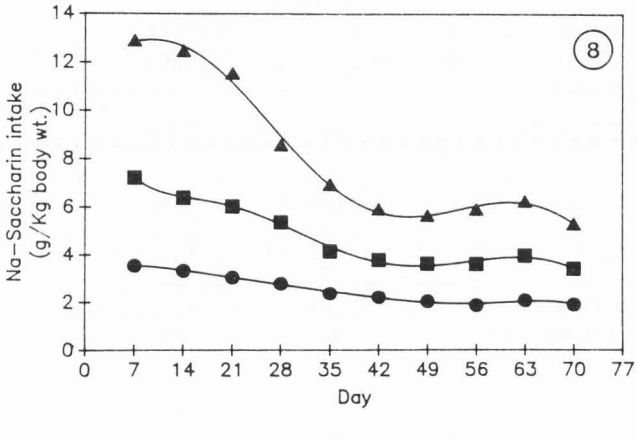


Figure 8. Consumption of sodium saccharin by the different groups expressed as g/kg body weight. 3.0%,; 5.0%,; 7.5%.

during weeks 2 and 10 and the total amount of sodium saccharin consumed per rat in each group are listed in Table 2. The consumption of food, and consequently sodium saccharin expressed as grams per kg. body weight, decreased during the course of the experiment at each dosage level (Fig. 8). Sodium saccharin caused a dose-related increase in water consumption (Table 2). A 4-7 gram increase in daily water intake occurred between successive doses of chemical, resulting in an overall 16 gram difference between the control and 7.5% sodium saccharin group. The cecum of rats fed sodium saccharin was enlarged, as observed previously [1, 15], and full and empty cecal weights increased significantly with each increasing dose of sodium saccharin (Table 2). The stomachs of rats fed sodium saccharin frequently showed hyperplastic and hyperkeratotic changes at the limiting ridge of forestomach and glandular stomach. These changes were present in 50-80% of rats fed 3.0% sodium saccharin at the 3 sacrifice periods and in all rats fed 5.0% or 7.5% sodium saccharin. It tended to increase in severity with increasing dose. However, there was little difference in severity between 4, 7, or 10 weeks at a given dose.

Table 3: Incidence of Light Microscopic Observations of the Urinary Bladder of Rats Fed Different Doses of Sodium Saccharin (NaS) for 4, 7, or 10 weeks

	Group	Level of NaS (%)	Number of Rats	Number of Rats with: Normal	Simple Hyperplasia: Mild	Simple Hyperplasia: Moderate	Papillary or Nodular Hyperplasia	Papilloma
At 4 Weeks	1	0	10	10(100%)	0	0	0	0
	2	3.0	10	10(100%)	0	0	0	0
	3	5.0	10	10(100%)	0	0	0	0
	4	7.5	10	5(56%)	4(44%)	0	0	0
At 7 Weeks	1	0	10	10(100%)	0	0	0	0
	2	3.0	10	10(100%)	0	0	0	0
	3	5.0	10	10(100%)	0	0	0	0
	4	7.5	10	0	3(30%)	6(60%)	1(10%)	0
At 10 Weeks	1	0	10	10(100%)	0	0	0	0
	2	3.0	10	10(100%)	0	0	0	0
	3	5.0	10	9(90%)	0	1(10%)	0	0
	4	7.5	10	0	2(20%)	4(40%)	3(30%)	1(10%)

Table 4: Classification by Scanning Electron Microscopy of Urinary Bladders of Rats Fed Different Doses of Sodium Saccharin (NaS) for 4 or 10 Weeks

	Group	Level of NaS (%)	Number of Bladders Examined	SEM Classification[b]: I	II	III	IV	V
At 4 Weeks	1	0	9[a]	4	5	-	-	-
	2	3.0	10	4	4	2	-	-
	3	5.0	7[a]	2	4	-	-	1
	4	7.5	10	-	1	2	3	4
At 10 Weeks	1	0	10	10	-	-	-	-
	2	3.0	10	1	3	5	1	-
	3	5.0	10	-	1	5	4	-
	4	7.5	10	-	1	-	3	6

[a]Fewer than 10 rats were examined in these groups because of difficulties in the processing of some bladders.
[b]Numbers represent rats in each group classified according to SEM criteria described in text.

Urinary Bladder Changes

The histologic changes observed in the urinary bladder are listed in Table 3. The criteria used for the various lesions are as described previously [4]. In tabulating the findings in the urinary bladder of each rat, only the most severe lesion observed was recorded. The bladder epithelium by light microscopy was normal in rats fed control diet, 3.0% sodium saccharin and, except one rat with moderate simple hyperplasia at 10 weeks, in rats fed 5.0% sodium saccharin. Rats fed 7.5% sodium saccharin in the diet had lesions which could be detected by light microscopy as early as 4 weeks. By 7 weeks, none of the rats fed 7.5% sodium saccharin had a normal bladder by light microscopy, and by 10 weeks, in addition to a 100% incidence of hyperplasia, one rat had a papilloma.

The changes observed by scanning electron microscopy are summarized in Table 4. The results for the examination at 4 and 10 weeks are given. The specimens collected at the 7 week interval could not be evaluated because of an electrical defect occurring during the critical point drying procedure which destroyed the specimens. The control rats at 10 weeks were all classified as having no abnormalities, whereas 5 of the 9 control rats at 4 weeks were placed in Class II. This might reflect the residual changes from the rapidly proliferating state observed in bladders during the first 3 weeks of life [2, 5, 8]. Nevertheless, these changes are minimal and are well within the range normally seen in this age and strain of rat. At 10 weeks, there were identifiable treatment-related changes in 9 of the rats fed 5.0% sodium saccharin and 6 of the rats fed

Table 5: The Labeling Index of the Urinary Epithelium in Male Rats Fed Various Doses of Sodium Saccharin (NaS) for 4, 7 or 10 Weeks

Group	Level of NaS (%)	Labeling Index of Urothelium[a]		
		4 Weeks	7 Weeks	10 Weeks
1	0	0.11±0.02(10)[b]	0.05±0.01(10)	0.07±0.02(10)
2	3.0	0.09±0.03(10)	0.17±0.06(10)	0.09±0.02(10)
3	5.0	0.08±0.02(9)	0.08±0.03(10)	0.20±0.06(10)[d]
4	7.5	0.36±0.10(5)[c]	1.11±0.41(10)[c]	0.64+0.21(8)[e]

[a]Mean ± S.E. [b]Number of rats evaluated in parentheses.

[c]Significantly greater than Groups 1, 2, and 3, p less than 0.001.

[d]Significantly greater than Group 1, p less than 0.05 by the least squares means procedure.

[e]Significantly greater than Groups 1, 2, and 3, p less than 0.01.

3% sodium saccharin. At both 4 and 10 weeks, most of the rats fed 7.5% sodium saccharin had markedly abnormal surface features as observed by scanning electron microscopy. The changes were more severe at 10 weeks than at 4 weeks at all 3 doses of sodium saccharin.

The labeling index of the different groups of rats at the 3 different time points are listed in Table 5. The labeling index of the bladder epithelium was markedly increased in all rats administered 7.5% sodium saccharin at all 3 time points, particularly at 7 weeks. This again may reflect a combination of accumulated changes and differences based on changes over time in dose as expressed in g/kg. Rats fed 5.0% sodium saccharin had an elevated labeling index only at the 10 week interval. Rats fed 3.0% sodium saccharin did not have a significantly different labeling index compared to the controls at any of the time intervals.

Discussion

The present study confirmed previous observations [3, 6, 9, 12] that sodium saccharin fed at high levels in the diet induces an urothelial proliferative response in the rat bladder, which is both dose and time dependent. The data from our study demonstrate that scanning electron microscopy is the most sensitive technique for detecting early proliferative changes in the rat urinary bladder, and that scanning electron microscopic observations can be semi-quantified to provide an evaluation of the effects of sodium saccharin. Scanning electron microscopy is considered particularly useful since half of a bladder or more can be readily examined, and it detects early changes in the urothelium at the level of a single cell. Sampling was not a problem, unlike what frequently happens with transmission electron microscopy. It would appear from our study that a useful short-term model for assessing the effects of various factors on sodium saccharin's ability to induce cell proliferation has been developed utilizing a four-week feeding protocol and observation by light and scanning electron microscopy, possibly supplemented with observations by autoradiography when indicated.

The number and size of the foci were increased in the sodium saccharin- treated rats, especially at the 7.5% level in the diet. Since the superficial epithelial cells provide a barrier between the urine and the underlying basal and intermediate cells, the increased extent of exposed cells in the bladders of rats fed high doses of sodium saccharin suggests that there is greater opportunity for exposure to components in the urine. Some of these urinary components are known to increase proliferation of epithelial cells.

Considerable data has accumulated that would suggest that the dose of sodium saccharin has to be quite high for any effect to be seen in the bladder epithelium, whether it is the tumorigenic response in a two-generation study [16], the promoting activity following initiation with N-butyl-N-(4-hydroxybutyl) nitrosamine [13], or the proliferative response in a short-term bioassay, such as described in this experiment. The data from this study suggest that there is a critical level in terms of g/kg dose of sodium saccharin for which a proliferative effect can be expected.

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Discussion with Reviewers

E. deHarven: What criteria do you currently use to delineate between uniform and pleomorphic microvilli, and, assuming that there is a clear cut identification of pleomorphic microvilli, is the number of pleomorphic microvilli per cell facilitating the differential diagnosis between simple hyperplasia and papillomatous proliferation?

Authors: Pleomorphic microvilli are distinguished from uniform microvilli predominantly on the basis of pleomorphism of length and shape. Thus, pleomorphic microvilli show variation in length and width individually and compared to each other. Terminal thickening (clubbing) is often seen and occasionally there are bifurcations in the microvilli. The number of pleomorphic microvilli per cell unfortunately does not distinguish between degrees of hyperplasia or between hyperplasia, papilloma, or carcinoma, although papillomas and papillary carcinomas tend to have more pleomorphic microvilli per surface area than hyperplastic lesions.

E. deHarven: The SEM changes observed at 4 weeks are primarily necrosis and exfoliation. How can you relate these changes with the hyperplastic response?

Authors: The changes at 4 weeks predominantly reflect focal necrosis. The increased labeling index and the later hyperplasia are considered to be indicative of a regenerative response to this cytotoxicity.

R. Oyasu: Does the presence of light microscopic changes signify the presence of advanced changes which are diffuse?

Authors: Although generally there is a correlation between severity of changes seen by SEM and those observed by light microscopy, this is not always the case. Some of this difference is due to different halves of the bladder being examined by the two methods and sampling difficulties in examining sections by light microscopy. The entire surface of the half examined by SEM is evaluated.

R. Oyasu: Please present data on the HPLC analysis of the diets.

Authors: HPLC analysis of the diets is given below:

Group	Stated Dose (%)	%[a] Dose determined by HPLC analysis Batch 1	Batch 2	Batch 3
1	0	0	0	0
2	3.0	2.8	2.9	2.5
3	5.0	4.7	4.7	4.5
4	7.5	7.3	7.3	6.7

[a]. % dose is an average of all the analyses done for each batch. Analyses were performed in duplicate from the top and bottom of each box.