Influences of Diet and Strain on the Proliferative Effect on the Rat Urinary Bladder Induced by Sodium Saccharin

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ABSTRACT

Rats were fed sodium saccharin as 5 or 7.5% of the diet by weight, and proliferation of the bladder epithelium was assessed by autoradiography, histology, and scanning electron microscopy. In Experiment 1, male F344 rats, 5 weeks old, were placed on a diet of 0, 5, or 7.5% NaS mixed in Prolab 3200, NIH-07, or AIN-76A diet for 4 or 10 weeks. In Experiment 2, 5-week-old F344 rats or 4-week-old Sprague-Dawley rats were fed 0, 5, or 7.5% NaS in Prolab 3200 or Purina 5002 diet for 10 weeks. In Experiment 1, at both the 4- and 10-week intervals, NaS had a greater effect on the urothelium when administered in the Prolab diet compared to the NIH diet, and there was little response with the AIN diet. Eight of 10 rats fed 7.5% NaS in Prolab 3200 for 4 or 10 weeks had bladders with simple or nodular hyperplasia, and eight of nine bladders contained abnormal surface features visible by scanning electron microscopy. At 10 weeks for control animals, the average labeling index following \[^{3}H\]thymidine incorporation into bladder epithelium was \(0.05\%\). For rats fed 7.5% NaS diets, the labeling index was 0.43% for Prolab, 0.14% for NIH-07, and 0.04% for AIN-76A. In Experiment 2, the response to NaS was considerably greater in F344 rats than in Sprague-Dawley rats fed the same diet, and for both strains, the response to NaS was greater in Prolab than in Purina diets. In conclusion, the proliferative effect of NaS on male rat urinary bladder depended on rat strain as well as on type of diet.

INTRODUCTION

Sodium saccharin administered at high levels in the diet acts as a carcinogen when administered over two generations (1) and as a tumor promoter for urinary bladder carcinogenesis when administered after initiation by a variety of agents (2, 3). Unlike classical carcinogens, it is not metabolized to a reactive electrophile, it does not react with DNA, and it is not mutagenic in a variety of in vitro and short-term assays (2, 3). However, increased proliferation in the urinary bladder epithelium of the rat (4-6) occurs within 1 week after administration of sodium saccharin begins (6). The urothelium is normally a mitotically quiescent tissue in the adult rat, with a labeling index of \(<0.1\%\) (6, 7). High doses of sodium saccharin, 5% of the diet, increase this by 4-10 times. This proliferative response is dose related (8).

Although the exact mechanism by which the proliferation rate of the bladder epithelium increases in response to sodium saccharin administration remains unknown, this effect has been shown to be dependent on the salt form in which the saccharin is administered in the diet (9). For example, potassium saccharin administered at a level in the diet similar to that of sodium saccharin significantly increases the proliferation rate above that of control rats, but the response is significantly less than that of sodium saccharin. There is no proliferative response if calcium saccharin or acid saccharin is administered to rats. Although there are differences in the solubility of these compounds, the urinary concentration of saccharin is not different following the administration of any of these salt forms. Nor is the ionic structure of the saccharin ion altered (10) when the different salt forms of saccharin are administered in the diet. There are variations in the concentrations of other ions in the urine, e.g., hydrogen, sodium, potassium, and calcium, as well as in urine volume and osmolality which might be involved in the proliferative response following the administration of high doses of sodium saccharin in the diet (4).

Most of the two-generation studies evaluating sodium saccharin as a carcinogen have been performed in Sprague-Dawley rats (2). These experiments have often utilized Purina rat chow as the diet. In contrast, F344 rats have frequently been used for short-term proliferative assays and for tumor promotion studies evaluating sodium saccharin (3, 4). A variety of diets have been fed in these experiments. Since several urinary parameters are influenced by dietary factors (11), it is likely that feeding various diets of different composition will result in differences in the urinary concentrations of several ions which might affect the proliferative response in the urinary bladder to sodium saccharin. The experiments described in this paper examined the influences of diet and strain on the proliferative effect of sodium saccharin on the rat urinary bladder.

MATERIALS AND METHODS

General Methods for Experiment 1 and Experiment 2

Sodium saccharin (99.9% pure) was provided by PMC Specialties Group, Inc. (Cincinnati, OH) from a single lot. Weanling, male F344 and Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Inc. (Kingston, NY). The F344 rats were 4 weeks old at the time of arrival, and the Sprague-Dawley rats were 3 weeks old. Upon arrival, the rats were weighed and randomly assigned to an experimental group (Tables 1 and 2) by a weight stratification method (12). They were kept in quarantine on their respective control diets for 1 week prior to study initiation. Rats were housed 5/cage on dry corncob bedding in polycarbonate cages (16 x 18 x 20 inches) with stainless steel wire bar covers (Lab Products, Inc., Maywood, NJ). Animal rooms were maintained at a temperature of 71 ± 5°F and 50 ± 20% humidity on a 12-h light, 12-h dark cycle. Food and distilled water were available ad libitum. Prolab 3200 diet was purchased from Agway, Inc. (St. Mary’s, OH) and Purina 5002 was purchased from Ralston-Purina Co. (St. Louis, MO). NIH-07 diet was prepared by Teklad, Inc. (Madison, WI) in accordance with the current NIH specifications and the published list of ingredients (13, 14). AIN-76A was prepared in our institute (14, 15). The nutrient composition of the different diets is tabulated in Table 3 from information published by the manufacturers (13-17). Diets used in this study were not analyzed for contaminants. However, significant levels of contaminants have not been found in Prolab 3200, Purina 5002, or NIH-07 diets that have been analyzed for other studies in our laboratory. Sodium saccharin was added to the diets at either 5 or 7.5% by weight, and the diets were then pelleted by Dyets, Inc. (Bethlehem, PA) for the Prolab, Purina, and NIH-07 diets, and within our institute for the AIN-76A diet. Diets were prepared monthly and were analyzed for sodium saccharin before feeding, using the high performance liquid chromatography procedure originally described by Tan and Pan (18), modified as described by Tibbels et al. (19).
Table 1 Design of Experiment 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Group</th>
<th>Dose of sodium saccharin (%)</th>
<th>4 wk sacrifice</th>
<th>10 wk sacrifice</th>
<th>For urine</th>
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<td>18</td>
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<td>10</td>
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</tr>
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</table>

Water consumption and diet consumption were measured over 7-day intervals, during Weeks 1 through 10 of the experiment. The rats were weighed at the beginning of the experiment and at the end of each consumption interval. One h before sacrifice, each rat was given an i.p. injection of [methyl-\textsuperscript{3}H]thymidine (1 \muCi/g body weight; New England Nuclear, Boston, MA) beginning at 9 a.m. and proceeding in numerical sequence, alternating among groups. With the rats under Nembutal anesthesia, the urinary bladder was inflated in situ with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The left side of each bladder was marked with hematoxylin following inflation, and then the bladder was removed and immediately placed in a container of the same fixative. The stomach was inflated in situ with 10% formalin, removed, and then placed in cacodylate buffer, pH 7.4. The left side of each bladder was marked with hematoxylin following inflation, and then the bladder was removed and immediately placed in a container of the same fixative. The stomach was inflated in situ with 10% formalin, removed, and then placed in the same fixative. The cecum was removed and weighed, cut open, and rinsed in saline solution, blotted gently, and weighed again. After fixation, the bladder was divided in half, and the right half was cut longitudinally into four strips to be used for histology and autoradiography. Glass slides were dipped in Kodak NTB-2 photographic emulsion, stored in light-proof boxes at 4°C for 3 weeks, and then developed with Kodak D19. The tissues were stained with hematoxylin and eosin. Bladder epithelial cells in all four strips were counted. One strip containing forearm and glandular stomach was processed with the bladder for histology and autoradiography as a positive control for the autoradiographic determination. The left half of each bladder was processed for SEM. The procedures for histopathological, autoradiographic, and SEM analyses were previously described (6, 9).

Experiment 1. One hundred and sixty-five male weanling F344 rats were distributed into 18 groups according to sacrifice interval and treatment as indicated in Table 1. The rats were fed appropriate experimental diets for 4 or 10 weeks. Urine and feces were collected during weeks 4 and 10 from Groups 6, 12, and 18. Rats were individually housed in mesh metabolism cages (Hazleton Systems, Aberdeen, MD) for 2 days prior to collection as described previously (11). Feces and urine were then collected separately for 24 h. Toluene was added to urine collection tubes as a preservative. The feces were weighed immediately after collection, dried, and reweighed to determine fecal moisture content. The dry weight and urine were analyzed for saccharin content (19). Urinary volume and pH were determined and the concentrations of sodium (20), potassium (20), calcium (21), and creatinine (22) were measured with a Beckman Astra 4 or 8 (Beckman Instruments, Inc., Brea, CA). Osmolality of the urine was measured by freezing point depression (21), using a Model 3011 Advanced Digmatic Osmometer (Advanced Instruments, Inc., Needham Heights, MA). As indicated in Table 1, different rats were utilized for urine collection experiments than were used for the tissue analyses. This was done to avoid excessive handling of the animals that were to be processed for tissue analysis.

Experiment 2. Fifty-eight F344 and 54 Sprague-Dawley male weanling rats were distributed into 12 groups according to treatment as indicated in Table 2. The rats were fed the appropriate test diet for 10

Table 2 Design of Experiment 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>Strain</th>
<th>Group</th>
<th>Dose of sodium saccharin (%)</th>
<th>No. of rats (10 wk sacrifice)</th>
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</thead>
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<tr>
<td>Prolab 3200</td>
<td>Sprague-Dawley</td>
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<tr>
<td></td>
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<td>6</td>
<td>7.5</td>
<td>10</td>
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<td>Purina 5002</td>
<td>Sprague-Dawley</td>
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<td>0</td>
<td>10</td>
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<td>Prolab 3200</td>
<td>F344</td>
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<td>5</td>
<td>10</td>
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<tr>
<td></td>
<td></td>
<td>12</td>
<td>7.5</td>
<td>10</td>
</tr>
</tbody>
</table>
day, was similar among diets at corresponding dietary concen-
diet. The consumption of sodium saccharin, expressed as g/kg/ 
regardless of the diet or the amount of sodium saccharin in the 
animals fed AIN-76A diet, which tended to consume slightly 
in NIH-07 diet compared to rats fed the respective control 
saccharin in all of the diets, and in rats fed 5% sodium saccharin 
rats fed NIH-07 diet was greater than of rats fed Prolab 3200 
are shown in Table 4. In control rats, the mean body weight of 
consumption, sodium saccharin consumption, and cecal weight 
less food, food consumption was similar in the various groups, 
weakening on the urinary bladder epithelium when administered in 
the Pro lact 3200 diet compared to the NIH-07 diet after 4 and 10 
trations. The amount of sodium saccharin consumed per rat 
over 10 weeks was less for rats fed AIN-76A diet than for rats fed 
Pro lact 3200 or NIH-07. Cecal weights were increased 
following sodium saccharin ingestion. The cecum was heavier 
in rats fed the control AIN-76A diet compared to rats fed 
control Pro lact 3200 or control NIH-07, and the difference 
between groups fed AIN-76A control and AIN-76A with so-
dium saccharin was less than with the other diets.

weeks. No urine or feces was collected in this experiment.
Statistical analyses of body and cecal weights and of autoradiography 
data were performed with a generalized linear model procedure from 
the Statistical Analysis System software package (SAS Institute, Inc., 
Cary, NC) (23). Duncan’s multiple-range test (24) was used for multiple

RESULTS

Experiment 1. The effects of sodium saccharin administration 
in the different diets on body weight, water consumption, food 
consumption, sodium saccharin consumption, and cecal weight 
are shown in Table 4. In control rats, the mean body weight of 
rats fed NIH-07 diet was greater than of rats fed Pro lact 3200 
diet. A lower body weight was evident in rats fed 7.5% sodium 
saccharin in all of the diets, and in rats fed 5% sodium saccharin 
in NIH-07 diet compared to rats fed the respective control 
diets. Water consumption was increased for rats ingesting either 
5 or 7.5% sodium saccharin in each diet. With the exception of 
animals fed AIN-76A diet, which tended to consume slightly 
60% less food, food consumption was similar in the various groups,
regardless of the diet or the amount of sodium saccharin in the 
diet. The consumption of sodium saccharin, expressed as g/kg/ 
day, was similar among diets at corresponding dietary concen-
trations. The amount of sodium saccharin consumed per rat 
over 10 weeks was less for rats fed AIN-76A diet than for rats fed 
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following sodium saccharin ingestion. The cecum was heavier 
in rats fed the control AIN-76A diet compared to rats fed 
control Pro lact 3200 or control NIH-07, and the difference 
between groups fed AIN-76A control and AIN-76A with so-
dium saccharin was less than with the other diets.

The changes in the urinary bladder in the various groups are 
shown in Table 5. This table includes the response to sodium 
saccharin assessed by labeling index, histopathology, and SEM. 
The three indices were generally affected in a dose-related 
manner by the administration of sodium saccharin in each diet.
In terms of an increase in labeling index and the appearance of 
hyperplasia, sodium saccharin had a considerably greater effect 
on the urinary bladder epithelium when administered in the 
Pro lact 3200 diet compared to the NIH-07 diet after 4 and 10 
weeks of treatment. When the bladders were evaluated by SEM, 
the bladders from the rats fed these two diets were classified 
"5" rather than "0-4", and were therefore classified as exhibiting 
the most severe epithelial changes. The number of animals in each classification is shown 
for each group.

\[\text{Histopathology} \]

\[\begin{array}{cccccc}
\text{Wk} & \text{Diet} & \text{Dose of sodium saccharin (%)} & \text{Labeling index} & \text{Histopathology} \\
\hline
4 & Pro lact & 0 & 0.11 ± 0.02 (10) & Normal & 0 \\
 & 7.5 & 0.62 ± 0.12 (10) & 2 & Simple hyperplasia & 1 \\
 & NIH-07 & 0 & 0.08 ± 0.02 (9) & 0 & Nodular hyperplasia & 5 \\
 & 7.5 & 0.24 ± 0.05 (9) & 6 & 1 & 3 \\
 & AIN-76A & 0 & 0.15 ± 0.01 (10) & 10 & 1 & 3 \\
 & 7.5 & 0.23 ± 0.07 (10) & 7 & 2 & 3 \\
10 & Pro lact & 0 & 0.05 ± 0.02 (10) & 10 & 6 & 3 \\
 & 7.5 & 0.43 ± 0.16 (9) & 2 & 1 & 5 \\
 & NIH-07 & 0 & 0.04 ± 0.01 (10) & 10 & 8 & 1 \\
 & 7.5 & 0.05 ± 0.01 (10) & 9 & 1 & 5 \\
 & AIN-76A & 0 & 0.04 ± 0.02 (10) & 10 & 6 & 3 \\
 & 7.5 & 0.03 ± 0.01 (10) & 9 & 2 & 3 \\
 & 7.5 & 0.04 ± 0.01 (9) & 6 & 1 & 1 \\
\end{array}\]

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manner by the administration of sodium saccharin in each diet.
In terms of an increase in labeling index and the appearance of 
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<table>
<thead>
<tr>
<th>Wk</th>
<th>Diet</th>
<th>Dose of sodium saccharin (%)</th>
<th>Labeling index (%)</th>
<th>Histopathology</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal Simple hyperplasia Nodular hyperplasia</td>
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<tr>
<td>4</td>
<td>Pro lab</td>
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<td>0.11 ± 0.02 (10)</td>
<td>10 0 0</td>
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<td>AIN-76A</td>
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<td>0.15 ± 0.01 (10)</td>
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<td>7.5</td>
<td>0.04 ± 0.01 (9)</td>
<td>6 1 1</td>
</tr>
</tbody>
</table>

* Mean ± SE; number of rats examined is given in parentheses.
* Data collected on a per cage basis so that SE per rat could not be determined; statistical analysis not performed since only 2 cages/group.
* Significantly different from rats fed NIH-07 diet without added sodium saccharin, P < 0.001.
* Significantly different from rats fed NIH-07 diet without added sodium saccharin, P < 0.001.
* Significantly different from rats fed NIH-07 diet without added sodium saccharin, P < 0.001.
* Significantly different from rats fed NIH-07 diet without added sodium saccharin, P < 0.001.

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Table 6: Urine and fecal determinations of rats fed sodium saccharin as 7.5% of different diets for 10 wk

<table>
<thead>
<tr>
<th>Diet</th>
<th>Volume (ml)</th>
<th>Sodium saccharin (mg/ml)</th>
<th>Na (meq/liter)</th>
<th>K (meq/liter)</th>
<th>Ca (mg/dl)</th>
<th>Osmolality (mosm/liter)</th>
<th>pH</th>
<th>Wet wt (g)</th>
<th>Dry wt (g)</th>
<th>Sodium Saccharin (mg)</th>
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</thead>
<tbody>
<tr>
<td>Prolab 3200</td>
<td>13.8 ± 0.6</td>
<td>48.9 ± 3.0</td>
<td>262 ± 16</td>
<td>134 ± 9</td>
<td>11.0 ± 1.0</td>
<td>1327 ± 93</td>
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<td>NIH-07</td>
<td>11.4 ± 1.2</td>
<td>50.8 ± 2.6</td>
<td>308 ± 16</td>
<td>134 ± 7</td>
<td>15.2 ± 2.3</td>
<td>1513 ± 72</td>
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<tr>
<td>AIN-76A</td>
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<td>71.2 ± 2.8</td>
<td>306 ± 14</td>
<td>72 ± 3̊</td>
<td>39.8 ± 2.7̊</td>
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<td>0.9 ± 0.2̊</td>
<td>75 ± 14̊</td>
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* Mean ± SE; 5 rats examined in each group.

10 weeks, by labeling index, histopathology, and SEM.

The various urinary and fecal chemical determinations of rats fed 7.5% sodium saccharin in the different diets are shown in Table 6. The urinary volume of rats fed sodium saccharin in Prolab 3200 was the greatest of the three diet groups. Nevertheless, the total amount of sodium saccharin excreted in the urine of all three groups was similar, due to a slightly greater concentration of saccharin in the urine of AIN-76A-fed rats. The osmolality of the urine did not differ significantly among groups nor was the sodium concentration different. However, the potassium concentration was significantly less and the calcium concentration was significantly greater in the urine of rats fed sodium saccharin in the AIN-76A diet compared to the other diets. The results for calcium were surprising in light of the lower calcium concentration in AIN-76A diet in comparison to the other diets. An increase in the urinary calcium concentration was not observed in a previous study (11) in which the effects of feeding 5% sodium saccharin for 10 weeks were compared in AIN-76A and Prolab 3200 diets. The urinary pH was lower in rats fed sodium saccharin in the AIN-76A diet compared to either Prolab 3200 or NIH-07. The amount of sodium saccharin excreted in the feces was similar for rats fed Prolab 3200 diet and NIH-07 diet, but it was markedly reduced in rats fed AIN-76A diet, largely due to the decreased fecal output of rats fed this diet.

Experiment 2. The body weight, water consumption, food consumption, and cecal weights of rats fed sodium saccharin in the different groups of this experiment are shown in Table 7. These values are for the 10-week time point, but the findings were similar over the entire course of the experiment. The F344 rats were considerably lighter than the Sprague-Dawley rats throughout the study. For both strains and both diets, rats fed sodium saccharin in the diet were lighter than rats fed control diet. Within each strain, rats fed Purina diet generally weighed more than rats fed Prolab 3200, but the difference was significant only for Sprague-Dawley rats fed control diet or 7.5% sodium saccharin diet. Water consumption was increased in rats fed sodium saccharin. Food consumption (g/rat/day) was nearly doubled in Sprague-Dawley rats compared to F344 rats, but was similar for all groups within the strains. Cecal weights increased with increasing dose of sodium saccharin in both strains and both diets.

The changes in the urinary bladder in the various groups of this experiment are shown in Table 8. Similar conclusions can be drawn from the data for labeling index, histopathology, and SEM. In general, the response to sodium saccharin was greater in F344 rats than in Sprague-Dawley rats fed the same type of diet. Also, for both strains, the response was greater in rats fed sodium saccharin in Prolab 3200 diet compared to Purina diet. One Sprague-Dawley rat fed Purina with 5% sodium saccharin had a bladder stone.

DISCUSSION

The results confirm that sodium saccharin induces a proliferative response in the urinary bladder of Sprague-Dawley and F344 rats when it is fed in one of a variety of diets. This proliferative response is apparent in an increase in \(^3\)Hthymidine labeling index, the development of hyperplasia, and epithelial changes. The proliferative response of the male rat urinary bladder
epithelium to high doses of sodium saccharin administered in the diet was influenced by the type of diet in which it was fed and by the strain of rat. Although there was a considerable effect in rats administered sodium saccharin in Prolab 3200 diet, there was little response in rats fed sodium saccharin in AIN-76A diet. The response in rats fed NIH-07 or Purina diet was intermediate. The increase in proliferation in F344 rats exceeded that in Sprague-Dawley rats. Sodium saccharin is representative of a large group of sodium salts known to act as tumor promoters for the male rat urinary bladder when high doses are administered in the diet. Evidence (3, 4, 9, 26) suggests that not just one but several urinary parameters must be affected for a proliferative response to occur following the administration of sodium salts in the diet. It appears that the urinary pH needs to be near neutral or above (generally pH >6.5) and the sodium concentration must be markedly elevated. It also appears that the urinary calcium level needs to be increased or the values observed with control diet. Increased urinary volume and decreased osmolality are also associated with the ingestion of these sodium salts and might also play a role in their proliferative effect in the bladder.

Although in Experiment 1 the urinary sodium concentration was similar among groups, there were differences in the urinary pH, calcium and potassium concentrations, and volume between rats fed AIN-76A and rats fed Prolab 3200 or NIH-07. These factors do not readily explain the differences that appeared between the Prolab 3200, the Purina, and the NIH-07 diets. Other factors must be involved in the response to sodium saccharin when it is administered in these different diets. As can be seen in Table 3, numerous differences exist in the composition of the diets, and some of these might influence the proliferative response of the bladder epithelium following sodium saccharin feeding. For example, dietary fat, which varies in these diets between 4.5 and 5.5%, has been shown to influence bladder carcinogenesis induced by acetylamino-fluorene in mice (27). It is important to note also that the sources of protein, carbohydrate, and fat vary among the diets, which also vary in the proportions and type of components of plant and animal sources (13-17).

Diet and strain have also been shown to influence sodium ascorbate tumor-promoting activity in male rats (28). The response to sodium ascorbate in two different laboratory chows differed depending on the strain of rat that was examined. In the present studies, it was apparent that strain also influenced the response to sodium saccharin. The F344 rat was more responsive than the Sprague-Dawley rat to the short-term proliferative effects of sodium saccharin. This finding corresponds to the differences in response of different strains of rats that were seen following long-term administration of sodium saccharin reported previously (29).

The influences of diet and strain have also been observed in studies of tumor promotion in other tissue systems. There is a difference in rat liver promotion by phenobarbital following diethylnitrosamine initiation, depending on whether a semipurified or nonpurified diet is used (30). Strain was also a critical factor in that study. Similarly, there can be a difference in response to tumor inhibitors depending on the diet used. Inhibition by retinyl acetate of mammary cancer induced by methylnitrosourea in female rats differs depending on the diet (31). The extent of tumor inhibition is greatest in rats administered retinyl acetate in Wayne diet compared to rats fed NIH-07 or AIN-76A diet (31). Extrapolating the proliferative, tumorigenic, and inhibitory properties of various chemicals from one animal study to another or from animals to humans therefore requires some caution. It is necessary to take into account the influences that the diet and strain used in a study might have on the physiological and metabolic responses to the test chemical.

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Influences of Diet and Strain on the Proliferative Effect on the Rat Urinary Bladder Induced by Sodium Saccharin

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