ABSTRACT

Sodium ascorbate, like other sodium salts such as saccharin, glutamate, and bicarbonate, produces urinary alterations when fed at high doses to rats, which results in mild superficial urothelial cytotoxicity and regeneration but not tumors in a standard 2-year bioassay. Sodium saccharin was shown to produce a low incidence of bladder tumors in rats if administered in a two-generation bioassay. In the present study, we evaluated sodium ascorbate in a two-generation bioassay that involved feeding to the male and female parental F344 rats for 4 weeks before mating, feeding the dams during gestation and lactation, and then feeding the weaned (at 28 days of age) male F1 generation rats for the remainder of their lifetime (up to 128 weeks of the experiment). Dietary levels of 1.0, 5.0, and 7.0% sodium ascorbate were tested. At 5.0 and 7.0% sodium ascorbate, there was an increase in urinary bladder urothelial papillary and nodular hyperplasia and the induction of a few papillomas and carcinomas. There was a dose-responsive increase in renal pelvic calcification and hyperplasia and inhibition of the aging nephropathy of rats even at the level of 1% sodium ascorbate. Because the short-term urothelial effects of sodium ascorbate in rats are inhibited by treatments producing urinary acidification to pH < 6.0, we coadministered high doses of long-term NH4Cl to groups of rats with 5.0 or 7.0% sodium ascorbate to evaluate the long-term effects. The combination of 7.0% sodium ascorbate plus 2.78% NH4Cl in the diet was toxic, and the group was terminated early during the course of the experiment. The group fed 5.0% sodium ascorbate plus 2.04% NH4Cl showed complete inhibition of the urothelial effects of sodium ascorbate and significant inhibition of its renal effects. We also demonstrated the presence of a calcium phosphate-containing urinary precipitate in rats fed sodium ascorbate at all doses, in a dose-responsive manner. The formation of the precipitate was inhibited by coadministration with NH4Cl. The proliferative effects of sodium ascorbate on the male rat urinary tract in this study are similar to those seen with sodium saccharin when administered in a two-generation bioassay. Mechanistic information suggests that this is a high-dose, rat-specific phenomenon.

INTRODUCTION

In 1974, it was reported (1) that sodium saccharin induced a significant incidence of bladder tumors in male rats when administered at high doses in the diet in a two-generation bioassay that involved administration to the parental F0 generation before conception, to the dams during gestation and lactation, and then to the pups, the F1 generation, for the remainder of their lifetime. This was confirmed in additional studies (2-4). A subsequent experiment showed that the administration of sodium saccharin beginning at birth produced incidences of bladder tumors similar to those in the two-generation bioassay (4). With the possible exception of one study (3) that began the feeding of sodium saccharin at 32 days of age and continued for more than 2 years, beginning the dosing after weaning (35-56 days of age) has not resulted in a significant incidence of bladder tumors (5-10).

On the basis of its carcinogenicity in rats, sodium saccharin was listed as a potential human carcinogen (5, 11, 12). Its use as a food additive was banned in Canada, and based on the Delaney amendment, efforts were made to ban its use in the United States. This was overridden by a Congressional moratorium allowing its continued use (11, 12); because this moratorium has been renewed periodically, the use of saccharin continues in the United States.

Extensive research since that time suggests that the carcinogenicity of sodium saccharin in rats is related to extensive physiological alterations in the urine that lead to the formation of a calcium phosphate-containing urinary precipitate, cytotoxic changes and regenerative hyperplasia of the urothelium, and ultimately bladder tumors (3, 5, 6, 13-18). The phenomenon seems to be specific to the rat, the response is greater in males than in females, and the process is dependent on administration of high doses with a no-observed-effect level of sodium saccharin at 1% of the diet (6, 19).

Several urinary parameters need to be at critical levels in sodium saccharin-fed rats for the precipitate to form and for tumors to be produced (6, 13-18). These include the high osmolality of rodent urine, an extremely high concentration of protein, adequate concentrations of calcium and phosphate, and a urinary pH level ≥ 6.5. Any treatment that produces urinary acidification, such as administration of the corresponding acid (acid saccharin; Ref. 20), coadministration of the sodium salt with high levels of NH4Cl (21), or administration in certain diets that yield acidic urine (e.g., the semi-synthetic AIN-76A diet; Ref. 22), completely inhibits the formation of the precipitate, the induction of urothelial cytotoxicity and regeneration, and, ultimately, the carcinogenic process (6, 13-15).

In 1979, Cohen et al. (23) reported that sodium saccharin enhanced the production of bladder tumors in mature male rats when it was fed after the initial administration of the bladder carcinogen N-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide. Subsequently, Fukushima et al. (24) reported that sodium ascorbate produced bladder tumors in this two-stage model after administration of N-butyl-N-(4-hydroxybutyl) nitrosamine. Sodium ascorbate and numerous other sodium salts fed at high dietary levels were shown to produce a precipitate in the urine similar to that formed after sodium saccharin feeding (16), along with similar urothelial toxicity and regenerative and tumorigenic properties (6, 13-18). Although most of those sodium salts have not been extensively evaluated, only the rat seems to develop urothelial proliferative responses to the feeding of high doses of the sodium salts, the response is greater in males than females, high dietary doses are required (generally, doses used are equimolar to ~5% sodium saccharin or higher), the results are negative for tumorigenicity in standard 2-year bioassays in rats, and the corresponding acid has no effect on the bladder (6, 13-18). In short-term tests in mature rats (4-13 weeks), these sodium salts produce urothelial hyperplasia (6, 13-18).

The other sodium salts that produce urothelial effects in rats similar to those of sodium saccharin are natural substances, either essential dietary ingredients (ascorbate, phosphate, and chloride) or components of cellular intermediary metabolism (glutamate, aspartate, succinate, and bicarbonate). None of these sodium salts has demonstrated tumorigenic activity when administered by itself without pretreatment with a carcinogen such as N-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide or N-butyl-N-(4-hydroxybutyl) nitrosamine, although potassium bicarbonate produced some bladder tumors in a 2.5-year study.
(25). However, only saccharin has been studied in a two-generation bioassay.

In preliminary studies (26), sodium ascorbate that was administered at high levels in the diet (4.86 or 6.84%) to parental rats before conception, to the dams during gestation and lactation, and then to the weaned pups through 15 weeks of age was shown to produce mild urothelial simple hyperplasia in the pups. Results were similar with sodium saccharin in the same study as in previous studies (27, 28). Coadministration of these sodium salts with appropriately high doses of NH₄Cl reduced urinary pH to <6 and inhibited the cytotoxic and proliferative responses in the offspring (26). In this report, we demonstrate that sodium ascorbate fed at high dietary levels to the male rat in a two-generation bioassay is tumorigenic to the bladder and that the urinary and urothelial effects are inhibited by coadministration with NH₄Cl.

MATERIALS AND METHODS

The protocol for the two-generation bioassay with sodium ascorbate corresponded to that used previously with sodium saccharin (4, 26). Use of animals in this study was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Ninety male and 180 female F344 rats were purchased from Charles River Breeding Laboratories, Inc. (Kingston, NY) at 4 weeks of age and kept in quarantine for 1 week. They were then randomized into six groups by a weight-stratification method, and their respective diets were begun (see Table 1). The procedures for treatment of rats, including the mating, feeding, weighing, culling, observations, and weaning, have been described previously in detail (26). The F₁ generation rats were weaned at 4 weeks of age, and the F₂ females were killed. Up to 40 to 54 days of age, the animals were treated on an individualized basis with respect to their date of birth. At that time, all animals were considered to be at week 0 of the F₁ generation portion of the experiment and were continued on their respective diets until the end of the experiment at 128 weeks. Fresh voided urine was collected in the early morning from rats in each group at routine intervals throughout the study to evaluate pH. During week 11, urine was collected for 24 h over ice from the first 10 animals in each group for assessment of urinary chemistries, including total ascorbate levels (ascorbate + dehydroascorbate; Ref. 29). At 14 weeks of age, all F₁ females were killed. Group 6 males were killed at this time because of the severe toxicity from the very high doses of NH₄Cl and the small number of rats remaining in the group.

During the course of the experiment, rats that were determined to be moribund based on weight loss and physical appearance were killed; surviving rats were killed at 128 weeks. At necropsy, the urinary bladder was inflated in situ with Bouin’s fixative, removed and placed in the fixative, rinsed in 70% ethanol within 4 h, and processed for paraffin embedding and light microscopic evaluation. All other organs were examined grossly and fixed in formalin. Other tissues that were routinely examined histopathologically included the forestomach, both kidneys, and tissues with grossly visible lesions. Lesions in the bladder were classified as described previously (30). Diagnoses of lesions in other tissues were based on standard classification systems (31).

RESULTS

No abnormalities were noted in the F₀ animals during feeding of the respective diets before conception and during lactation, nor was there any evidence of increased morbidity or mortality. However, there was a decreased percentage of successful matings that resulted in live litters in Groups 3–6, especially in Group 6. There were no differences among groups in body weights of the pups at birth, but by the time of weaning, Groups 4 and 6 (coadministered NH₄Cl) showed significantly lower weights than the other groups (Table 1). By week 0 of the experiment, rats fed 7.0% sodium ascorbate (Group 5) weighed significantly less than the controls, and by 24 weeks the rats fed 5.0% sodium ascorbate (Group 3) weighed significantly less than the controls. Average weights at various periods of the experiment through week 78 are presented in Table 1. After that time, the weights of animals with leukemia grossly distorted the average weights in the groups.

Among groups, the animals ate approximately the same amount of food during the course of the experiment (Table 1), but there was increased water consumption in a dose-related fashion for the various sodium ascorbate groups, as would be expected with increased sodium ingestion. The coadministration of NH₄Cl did not affect food or water consumption.

Except for Group 6, mortality was not increased in any group compared with controls. However, in Group 4, there was significantly decreased mortality during the course of the experiment. The most common cause of death in all groups was leukemia, with grossly enlarged spleen and usually diffuse infiltrates of leukemic cells in other tissues, especially the liver and lungs. In the various groups, the incidences of tumors except for those in the bladder were those seen typically in this strain of rats (31). There were no significant differences among groups in incidences of these other tumors except for Group 4, in which the incidences were reduced (data not shown). It is likely that the decreased tumor incidences and decreased mortality in this group are secondary to the decreased weight gain (32).

Fresh voided urine was collected during weeks 4 and 11 and analyzed for calcium phosphate-containing urinary precipitate (Fig. 1; Refs. 16–18). After 4 weeks, the number of rats...
hyperplasia also had simple hyperplasia. These results confirm previous observations (26) showing a dose response and the prevention of the formation of the precipitate by coadministration with NH₄Cl.

There was little difference in urinary pH among groups except for the animals receiving NH₄Cl, although the pH tended to decrease slowly during the course of the experiment. The doses of NH₄Cl chosen for this experiment produced a urinary pH consistently below 6.3, usually <6.0 in Groups 4 and 6.

Urinary ascorbate levels increased in a dose-responsive manner (Table 2), although clearly the increases in urinary ascorbate did not correspond to the large amounts in the diets. This is likely related to rats' ability to synthesize ascorbate and metabolize it differently than humans. The urinary concentrations of ascorbate were considerably lower than those of saccharin (200–300 mm) after comparable doses of the sodium salts in the diet. The concentrations of other urinary constituents (Table 2) are as expected for rats with increased sodium and water consumption with correspondingly increased urinary volumes and also as expected for rats given high doses of NH₄Cl (33).

Incidences of proliferative lesions in the bladder are listed in Table 3. Few papillomas or carcinomas were induced, but there was an increase in papillary/nodular hyperplasia, a preneoplastic lesion of the rat urinary bladder, in the 5.0 and 7.0% sodium ascorbate groups in a dose-responsive manner. Coadministration with NH₄Cl completely inhibited the development of proliferative lesions in the urinary bladder.

Calcification of the fornix of the renal pelvis extending into the covering epithelium of the renal papilla and renal pelvis and urothelial

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>pH</th>
<th>Volume (ml)</th>
<th>Ascorbic acid (mm)</th>
<th>Creatinine (mg/dl)</th>
<th>Osmolality (mOsm/liter)</th>
<th>Protein (mg/ml)</th>
<th>Sodium (mEq/liter)</th>
<th>Potassium (mEq/liter)</th>
<th>Calcium (mg/dl)</th>
<th>Magnesium (mg/dl)</th>
<th>Phosphorus (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>6.8 ± 0.2</td>
<td>9 ± 0.4</td>
<td>0.1 ± 0.0</td>
<td>111 ± 3.8</td>
<td>2226 ± 61</td>
<td>2.3 ± 0.07</td>
<td>143 ± 7</td>
<td>388 ± 12</td>
<td>11.8 ± 0.6</td>
<td>20.1 ± 1.0</td>
<td>188 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>1% sodium ascorbate</td>
<td>7.1 ± 0.1</td>
<td>9 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>101 ± 3.0</td>
<td>2149 ± 92</td>
<td>2.1 ± 0.05</td>
<td>216 ± 6</td>
<td>351 ± 18</td>
<td>11.8 ± 0.7</td>
<td>17.4 ± 0.9</td>
<td>190 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>5% sodium ascorbate</td>
<td>7.6 ± 0.2</td>
<td>16 ± 1.3</td>
<td>1.4 ± 0.2</td>
<td>57 ± 4.5</td>
<td>1707 ± 77</td>
<td>1.4 ± 0.10</td>
<td>295 ± 19</td>
<td>189 ± 11</td>
<td>5.3 ± 0.5</td>
<td>14.8 ± 1.2</td>
<td>135 ± 11</td>
</tr>
<tr>
<td>4</td>
<td>5% sodium ascorbate + 2.04% NH₄Cl</td>
<td>5.7 ± 0.1</td>
<td>15 ± 1.0</td>
<td>2.7 ± 0.3</td>
<td>58 ± 2.9</td>
<td>1974 ± 40</td>
<td>1.5 ± 0.05</td>
<td>330 ± 6</td>
<td>210 ± 6</td>
<td>27.0 ± 1.0</td>
<td>45.6 ± 3.2</td>
<td>217 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>7% sodium ascorbate</td>
<td>7.8 ± 0.1</td>
<td>19 ± 0.9</td>
<td>2.9 ± 0.4</td>
<td>46 ± 0.7</td>
<td>1282 ± 26</td>
<td>1.2 ± 0.04</td>
<td>322 ± 11</td>
<td>151 ± 3</td>
<td>4.0 ± 0.5</td>
<td>15.8 ± 0.9</td>
<td>123 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>7% sodium ascorbate + 2.78% NH₄Cl</td>
<td>5.8 ± 0.1</td>
<td>16 ± 0.7</td>
<td>3.9 ± 0.3</td>
<td>47 ± 2.9</td>
<td>1897 ± 42</td>
<td>1.2 ± 0.06</td>
<td>362 ± 14</td>
<td>176 ± 6</td>
<td>32.3 ± 1.9</td>
<td>50.1 ± 2.5</td>
<td>191 ± 10</td>
</tr>
</tbody>
</table>

*Significantly different from controls (P < 0.05).
**Significantly different from the 5% sodium ascorbate group (P < 0.05).
***Significantly different from the 7% sodium ascorbate group (P < 0.05).

Table 3 Histopathology of the urinary bladder and kidney of F1 male rats in a two-generation bioassay of sodium ascorbate and the effects of urinary acidification by coadministration of NH₄Cl

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Urinary bladder</th>
<th>Kidney</th>
<th>Pelvic calcification</th>
<th>Pelvic hyperplasia</th>
<th>Aging nephropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>58 50 8</td>
<td>1</td>
<td>56 2 12</td>
<td>9 49</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1% Sodium Ascorbate</td>
<td>60 42 18</td>
<td>2 1 49</td>
<td>11 34</td>
<td>7 27 18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5% Sodium Ascorbate</td>
<td>45 26 19</td>
<td>5 1 11</td>
<td>34</td>
<td>7 38</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5% Sodium Ascorbate + 2.04% NH₄Cl</td>
<td>41 39</td>
<td>2 1 30</td>
<td>11 32</td>
<td>9 13 28</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7% Sodium Ascorbate</td>
<td>52 24 28</td>
<td>9 2 6</td>
<td>46 3 49</td>
<td>47 5</td>
<td></td>
</tr>
</tbody>
</table>

Based on the number of rats living at week 52 of the experiment when the first case of papillary/nodular hyperplasia was diagnosed.

Bladder epithelium was entirely normal. All rats with papilloma or carcinoma also had papillary/nodular hyperplasia and simple hyperplasia, and all rats with papillary/nodular hyperplasia also had simple hyperplasia.

*Significantly different from controls [Group 1 (P < 0.05)].
**Significantly different from controls (P < 0.05).
***Transitional carcinoma of the renal pelvis. Bladder histology was normal.

Significantly different from rats fed 5% sodium ascorbate [Group 3 (P < 0.05)].

0 = none; + = fornix; ++ = fornix + papillia and/or pelvis; +++ = extensive.

0 = none; + = mild; ++ = moderate; +++ = severe.
papillary and nodular hyperplasia were increased in frequency and in severity in the rats given sodium ascorbate in a dose-responsive manner (Table 3). Aging nephropathy, commonly found in the male rat, was present in controls but was decreased in severity in a dose-responsive fashion in the rats treated with sodium ascorbate. All of the renal effects produced by sodium ascorbate were mitigated by coadministration of NH₄Cl.

A few instances of hyperplasia at the limiting ridge between the forestomach and glandular stomach were observed in each group, and there was one squamous cell carcinoma of the forestomach in Group 2. This is in contrast to the hyperplasia at the limiting ridge always seen in rats given saccharin (34). Also, there was no evidence in the present experiment of the cecal enlargement commonly associated with high dietary doses of sodium saccharin in rats (4).

**DISCUSSION**

Administration of high doses of the sodium salt of ascorbate or saccharin to rats, particularly males, leads to formation of a calcium phosphate-containing precipitate that is cytotoxic to the superficial layer of the bladder epithelium, leading to regenerative hyperplasia (6, 13-18). Formation of the precipitate and the resulting cytotoxicity, regenerative hyperplasia, and tumorigenicity in a two-stage model of carcinogenesis have been qualitatively similar for the various sodium salts that have been studied (6, 13-18). Differences in potency for the various sodium salts have been considerable with respect to both proliferative responses in short-term (<2 weeks) studies and 2-stage tumorigenicity studies. However, this is only the second of these sodium salts to be administered in a two-generation bioassay. With sodium saccharin, tumor incidences in male rats in various two-generation bioassays have been 0, 9, 27, and 47% at the 5% dietary dose, and 16 and 17% at the 7.5% dose. Although the tumorigenic response seen in the present study with sodium ascorbate was quantitatively less than that seen with sodium saccharin (1-5), the results with sodium ascorbate showed similar increased incidences of proliferative lesions, including papillomas and carcinomas, in the bladder at the end of the feeding period in the F₁ generation of male rats. Bladder papillomas and carcinomas occur spontaneously extremely rarely in this strain of rats (31, 32). Formation of the urinary precipitate by the feeding of sodium salts is prevented by the acidification of urine below pH 6.3 (16-18, 26); after sodium ascorbate administration in the present experiment, the formation of the urinary precipitate and the induction of proliferative lesions of the bladder were inhibited. Similarly, NH₄Cl mitigated the renal effects produced by sodium ascorbate.

Formation of the cytotoxic, calcium phosphate-containing precipitate seems to be an essential step in the carcinogenicity of sodium salts in the male rat (19). It is a high-dose phenomenon, and it also seems to be specific for the rat. The specificity in rats seems to be related to the extraordinarily high protein concentrations in the urine, with levels higher in male than in female rats because of the presence of α₂u-globulin (6, 35, 36). A sustained urinary pH ≥ 6.5 in rats that are fed high doses of these sodium salts also plays an important role. Although the urinary osmolality is less in rats fed the sodium salts compared with controls (a difference associated probably with increased water ingestion), the overall osmolality in these rats is considerably higher than the osmolality usually seen in humans (6, 13-15, 33). Also, in rats fed these sodium salts, there is an increased excretion of mucopolysaccharides, the major organic component of the precipitate (6, and unpublished observations).³

When fed sodium salts, mice seem unable to form this precipitate or to produce a proliferative or tumorigenic response, despite the fact that the urinary pH is above 7.0 and the protein levels and osmolality are comparable to those in the rat (33, 37). However, in mice, there are significantly lower urinary concentrations of both calcium and phosphate, which affect the physical chemical aspects of the precipitation of calcium phosphate (33, 37). Monkeys and humans have considerably lower urinary concentrations of protein (33, 38) and markedly lower urinary osmolality (33). Monkeys do not form the calcium phosphate-containing precipitate when fed sodium saccharin, and in an experiment recently completed at the National Cancer Institute, monkeys that for 18-23 years were fed sodium saccharin at a level 5-10 times the suggested allowable daily intake showed no evidence of urinary precipitate and no evidence of cytotoxic or proliferative changes in the urinary bladder epithelium (39). Similarly, in humans, numerous epidemiological studies have generally not shown evidence of significantly increased incidences of bladder cancer (40) or development of hyperplastic (41) lesions in individuals exposed to sodium saccharin. The effects of vitamin C in bladder carcinogenesis in humans are unknown, but the evidence, although quite limited, thus far does not suggest that it is carcinogenic to the human bladder (42).

The evidence from the present study indicates that sodium ascorbate, like sodium saccharin, produces bladder proliferative lesions in male rats when administered at high dietary levels in a two-generation protocol. On the basis of mechanistic understanding of the process and extensive epidemiological studies, it is highly unlikely that sodium ascorbate, sodium saccharin, or other sodium salts pose a significant bladder cancer risk to humans.

Although animal models are useful in delineating various aspects of carcinogenesis with respect to the human situation, it is essential that differences between the animal models and humans be understood (43-45). In circumstances such as with these sodium salts, the rat model is inappropriate for extrapolation to humans. Thus, the absolute restrictiveness posed by the Delaney Amendment cannot be scientifically supported (46).

**ACKNOWLEDGMENTS**

We gratefully acknowledge the critical review and discussions provided by Drs. Clifford Chappell and William Berndt, the technical assistance of Margaret St. John and Marty Cano, and the secretarial assistance of Denise Miller and Michelle Moore.

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