ABSTRACT

The existence of at least two stages in bladder carcinogenesis was evaluated in male Fischer rats using N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) fed for six weeks at a level of 0.2% of the diet as the initiator. Sodium saccharin and DL-tryptophan were fed at levels of 5 and 2% of the diet, respectively, as possible promoting chemicals, and they were fed either immediately after FANFT administration or after six weeks of FANFT plus six weeks of control diet. All surviving rats were killed at the end of two years. Both chemicals significantly increased the incidence of bladder tumors following FANFT feeding compared to six weeks of FANFT feeding followed by control diet, and the results were similar whether saccharin or tryptophan feeding was started immediately after FANFT feeding was concluded or after a six-week delay. Saccharin was considerably more potent as a promoting agent than was tryptophan, inducing higher incidences of bladder tumors and having a shorter latent period. Long-term administration of FANFT induced a 100% incidence of bladder cancer. Sequential epithelial changes were observed by scanning and transmission electron microscopy as well as by light microscopy. Pleomorphic microvilli were present on the superficial cells of all tumors examined and on the surface cells of hyperplastic bladder epithelium after six weeks of FANFT plus six weeks of saccharin, but not after six weeks of FANFT and six weeks of control diet. Rats fed only saccharin, tryptophan, or control diet did not have bladder tumors or pleomorphic microvilli on bladder epithelium. These data suggest that saccharin and tryptophan might act as tumor-promoting agents during bladder carcinogenesis.

INTRODUCTION

We have focused our attention for 10 years on developing and defining an experimental model for studying the pathogenesis of bladder cancer, particularly the early stages (20, 27, 63). The Fischer rat, an inbred strain, has been found to be an appropriate animal, and the nitrofuran FANFT* has proved to be an effective organ-specific carcinogen, inducing a 100% incidence of bladder cancer after a relatively short time when given p.o. For the last few years, we have been particularly concerned with the identification of possible morphological markers of neoplastic change in bladder epithelium, using light microscopy and both scanning and transmission electron microscopy. This required a model in which the sequence of biological and pathological events was predictable. In a previous study, we identified as "subthreshold" a dose of FANFT, 0.2%, which when fed for 6 weeks produced no bladder tumors if the rats were then fed control diet until 84 weeks, although the same dose produced tumors if fed for 8 weeks followed by control diet (34). The present investigation was based on the concept that this subthreshold dose of FANFT, an "incomplete carcinogen" at the level of administration, could serve as an initiator, permitting us to explore the phase of tumor promotion in this model system.

This concept, the use of minimal amounts of known chemical carcinogens as initiating agents and the subsequent administration of agents known to be noncarcinogenic when administered by themselves, has been used by investigators for many years in their attempts to identify various stages in the carcinogenic process (67), beginning with investigations of 2-stage murine skin carcinogenesis (8-10). A similar process has subsequently been demonstrated in other tissues, notably the liver (47).

For the urinary bladder (29, 31, 32), it has been shown by Hicks that instillation into the rat bladder of a subcarcinogenic dose of MNU followed by p.o. administration of saccharin or cyclamate resulted in a high incidence of bladder cancer, while saccharin or cyclamate alone induced a very low incidence of bladder cancer. Cyclophosphamide, known to induce necrosis and then a marked hyperplasia of the bladder epithelium in rats, did not induce bladder tumors in the same experimental system after a subcarcinogenic dose of MNU.

In the experiments described in this report, we attempted to determine whether at least 2 stages existed in bladder carcinogenesis, using for initiation an agent would could be given in the diet rather than instilled into the bladder and as promoting agents in this system, saccharin and tryptophan. Saccharin, administered in the diet rather than in the drinking water, was used because of its demonstrated effectiveness as a promoting agent in the MNU model as described above. Tryptophan was chosen as another possible promoting agent because of the suggested relationship between abnormal tryptophan metabolite levels and bladder cancer in humans (14, 48, 68) and because of a variety of studies in experimental animals indicating a possible relationship of tryptophan metabolites with bladder cancer (14, 48).

Previous studies in our laboratory on the FANFT bladder cancer model in Fischer rats indicated that an early marker
of irreversibility of proliferative epithelial lesions was the presence of pleomorphic microvilli on the luminal surfaces of epithelial cells as detected by scanning electron microscopy (20, 27, 33, 34). Similar findings have been reported by others using different carcinogens administered to other strains of rats (3, 30), and comparable changes have been reported in humans with bladder tumors (28, 35). We therefore utilized scanning electron microscopy and light microscopy in these studies to correlate morphological findings with biological events.

MATERIALS AND METHODS

Male Fischer rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.), and were 4 weeks of age at the beginning of the experiment. They were housed 4/cage, were maintained at 24° and 50% humidity on a 12-hr light-dark cycle, and had food and water available ad libitum. FANFT (Saber Laboratories, Morton Grove, III.), sodium saccharin (Sigma Chemical Co., St. Louis, Mo.), and DL-tryptophan (Sigma) were mixed in a powered diet (Charles River rat chow) at doses of 0.2, 5.0, and 2.0% by weight, respectively. A sample of the sodium saccharin was evaluated for the presence of ortho- and para-toluenesulfonamide by M. C. Bowman (National Center for Toxicological Research, Jefferson, Ark.). No ortho-toluenesulfonamide was detected (limits of detection, 0.03 ppm), and approximately 0.03 ppm of what was considered to be para-toluen-sulfonamide was detected.

The rats were divided into 11 groups as illustrated in Chart 1. Saccharin and tryptophan were begun immediately after 6 weeks of FANFT (Group 1) or after a 6-week delay during which time they received control diet (Group 2). Group 3 received control diet for 6 weeks and were then fed diet containing saccharin. Saccharin was discontinued in Groups 1 to 3 at the end of the 83rd week of the experiment because some of the rats in Groups 1 and 2 had developed severe hematuria and had bladder tumors. They received control diet until the end of the experiment. Rats fed DL-tryptophan (Groups 4 to 6) continued to receive the chemical in their diet until the end of the experiment at 104 weeks. Group 7 was the control group and was fed control diet for the entire length of the experiment. Rats in Group 8 were fed FANFT for 6 weeks followed by control diet until the end of the experiment. Group 9 was fed FANFT for 6 weeks followed by 6 weeks of control diet, 30 weeks of FANFT, and then control diet until they died. Group 10 was fed control diet for 6 weeks, followed by 36 weeks of FANFT (same total period of FANFT administration as in Group 9), and then control diet until they died. Rats in Group 11 received FANFT for the entire time they were alive in the experiment. All of the rats in Groups 9 to 11 died due to bladder cancer by the end of Weeks 78, 80, and 77 of the experiment, respectively.

All rats were weighed at the beginning of the experiment and at the end of Weeks 2, 6, 7, 9, and 12 and monthly thereafter. Food consumption was determined at the same intervals. When a rat was found dead or was killed, a complete autopsy was performed and the tissues, except the bladder, were fixed in 10% buffered formalin and embedded in paraffin; sections were then stained with hematoxylin and eosin. When indicated, sections were stained with other stains. The urinary bladders were inflated with Bouin’s fixative, washed 3 to 5 times in 70% ethanol, and then processed as above.

The number of rats in each group at the start of the experiment is listed in Table 1. An additional 4 rats were sacrificed from each of Groups 7 and 8 at the end of the sixth week of the experiment and from each of Groups 1, 3, 4, 6, 7, 8, and 11 at the end of the 12th week to examine for early lesions in the bladder. These rats are not tabulated as part of these groups in Tables 1 to 3. The bladders from 2 of the 4 rats from each group at each time interval were inflated with Bouin’s fixative and processed for light microscopy as described above. The bladders from the other 2 rats from each group were inflated transurethrally through a 25-gauge needle with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, under a constant pressure of 50 mm of mercury to a volume of 0.4 to 0.5 ml, depending on the size of the rat, and processed for light, transmission, and scanning electron microscopic examination as described previously (20, 33). Two or more rats from each group were similarly examined at the end of the experiment, and the remainder were processed only for light microscopy as described above.

RESULTS

The rats in all groups gained weight at a rate similar to the control group except for the rats fed DL-tryptophan which gained at a rate approximately 10% less than the controls (Table 1). Rats in Groups 9 to 11, those on long-term FANFT feeding, grew at rates comparable to those of the controls until after 52 weeks at which time several rats had gross hematuria due to large bladder tumors, and the rats gradually lost weight. A similar process was observed in rats in Groups 1 and 2 after 76 weeks, but not all rats in these 2 groups showed such changes. Most of the rats fed tryptophan alone or after FANFT (Groups 4 to 6), saccharin without previous FANFT (Group 3), or FANFT for only 6 weeks (Group 8) survived until the end of the experiment (Table 1). All of the rats in Groups 9 to 11, which received
long-term administration of FANFT, died by the end of
Weeks 78, 80, and 77, respectively, due to bladder cancer. Several of the rats prefed FANFT and then fed saccharin (Groups 1 and 2) also died due to bladder cancer before the end of the experiment. The decreased number of rats in the control group (Group 7) at the end of the experiment was due to sequential killing of rats during the experiment for electron microscopic evaluation; none had bladder cancer. All of the rats fed saccharin (Groups 1 to 3) had intermittent diarrhea which ceased upon discontinuance of saccharin in the diet.

The estimated average cumulative consumption of each chemical is shown in Table 1 as g/rat/total time of administration of the chemical. It represents a maximum estimate inasmuch as no attempt was made to account for spillage, and it is estimated for the rats surviving the entire length of the feeding period. Generally, the rats fed saccharin in their diet consumed approximately 20% more food than did the control group or rats fed the other chemicals. The relative consumptions of the chemicals were approximately 2.5 to 3.0, 0.8 to 1.2, and 0.07 to 0.12 g/kg for saccharin, tryptophan, and FANFT, respectively, with the relative consumption gradually decreasing as the rats grew.

Bladder lesions observed in each group are summarized in Table 2. Each rat is tabulated in the column of the most advanced lesion found in the bladder. Usually, rats with papillomas or cancer also had areas of hyperplasia in the bladder. Tumors which were benign by histological criteria were designated as papillomas. A diagnosis of cancer was based on the loss of differentiation to the surface, presence of nuclear pleomorphism, and the presence of mitoses. The presence or absence of invasion was determined microscopically, and if questionable the lesion was classified as noninvasive. The extent of the bladder lesions in the various groups is shown in Table 3. Bladders having a carcinoma and sarcoma were classified as to extent of invasion of only the epithelial component since the sarcoma presumably originated in the submucosa. The sarcomas were usually anaplastic, spindle cell tumors histologically and usually invaded through the muscle wall. Statistical comparison of incidences was performed using the exact method for 2 x 2 tables.

Rats fed only the control diet, only saccharin (Group 3), or only tryptophan (Group 6) had no lesions of the urinary bladder except for occasional instances of mild, simple epithelial hyperplasia with the mucosa 4 to 5 cell layers thick rather than the usual 3. The group fed FANFT for 6 weeks followed by control diet (Group 8) included one rat with a papilloma and 4 with carcinomas, one of which showed microscopic invasion of the submucosa. These tumors were small, and there was only one tumor in each affected bladder. In contrast, the rats fed FANFT for long terms (Groups 9 to 11) all had bladder carcinomas, several had invasive lesions (Tables 2 and 3), and the carcinomas were usually large and often multiple.

Rats fed FANFT for 6 weeks and then fed saccharin, either immediately after the FANFT feeding (Group 1) or after a 6-week delay (Group 2), developed high incidences of bladder cancer (p << 0.001 and < 0.002, respectively, as compared to Group 8), and several cancers were invasive. None of the rats in these 2 groups had normal bladders. Rats prefed FANFT for 6 weeks and then fed tryptophan, either immediately after the FANFT feeding (Group 4) or after a 6-week delay (Group 5), also had significantly increased incidences of bladder carcinomas (p < 0.04 and < 0.05, respectively, as compared to Group 8), but the incidences were lower than with saccharin and the carcinomas were less commonly invasive. If the incidences of all bladder tumors (papillomas plus cancers) in the groups receiving FANFT and tryptophan were compared to the incidences in the group receiving 6 weeks of FANFT followed by control diet (Group 8), the differences also were signifi-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cumulative consumption of chemicals and growth and survival of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>No. of rats at start</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------</td>
</tr>
<tr>
<td>1. FANFT → saccharin</td>
<td>20</td>
</tr>
<tr>
<td>2. FANFT → control → saccharin</td>
<td>20</td>
</tr>
<tr>
<td>3. Control → saccharin</td>
<td>20</td>
</tr>
<tr>
<td>4. FANFT → tryptophan</td>
<td>20</td>
</tr>
<tr>
<td>5. FANFT → control → tryptophan</td>
<td>20</td>
</tr>
<tr>
<td>6. Control → tryptophan</td>
<td>20</td>
</tr>
<tr>
<td>7. Control</td>
<td>42</td>
</tr>
<tr>
<td>8. FANFT → control</td>
<td>20</td>
</tr>
<tr>
<td>9. FANFT → control</td>
<td>16</td>
</tr>
<tr>
<td>10. Control → FANFT → control</td>
<td>20</td>
</tr>
<tr>
<td>11. FANFT</td>
<td>42</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D.

$^b$ For groups receiving 2 chemicals, the average cumulative dose given first is the amount of FANFT consumed. The average cumulative dose represents the average amount of the chemical consumed per rat for the length of the experiment for the rats surviving the entire period of feeding the chemical.

$^c$ Numbers in parentheses, percentage.
Lesions of the urinary bladder and other tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>Effective no. of rats</th>
<th>Normal</th>
<th>Simple hyperplasia</th>
<th>Nodular or papillary hyperplasia</th>
<th>Papilloma</th>
<th>Cancer</th>
<th>Calcification in bladder lesions</th>
<th>Interstitial cell tumors of testis</th>
<th>Tissue</th>
<th>Histology</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. FANFT → saccharin</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>18</td>
<td>6</td>
<td>13</td>
<td>Islet cell</td>
<td>Adenoma</td>
<td>1</td>
</tr>
<tr>
<td>2. FANFT → control → saccharin</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>13</td>
<td>1</td>
<td>9</td>
<td>Islet cell</td>
<td>Adenoma</td>
<td>2</td>
</tr>
<tr>
<td>3. Control → saccharin</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td></td>
<td>Islet cell</td>
<td>Adenoma</td>
<td>2</td>
</tr>
<tr>
<td>4. FANFT → tryptophan</td>
<td>19</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>13</td>
<td>Abdomen</td>
<td>Fibroma</td>
<td>1</td>
</tr>
<tr>
<td>5. FANFT → control → tryptophan</td>
<td>20</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>1</td>
<td>13</td>
<td>Islet cell</td>
<td>Adenoma</td>
<td>1</td>
</tr>
<tr>
<td>6. Control → tryptophan</td>
<td>19</td>
<td>16</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td></td>
<td>Lung</td>
<td>Adenoma</td>
<td>1</td>
</tr>
<tr>
<td>7. Control</td>
<td>42</td>
<td>37</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td></td>
<td>Epididymis</td>
<td>Adenoma</td>
<td>1</td>
</tr>
<tr>
<td>8. FANFT → control</td>
<td>20</td>
<td>5</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>17</td>
<td>Abdomen</td>
<td>Fibroma</td>
<td>2</td>
</tr>
<tr>
<td>9. FANFT → control → FANFT → control</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>Ear duct</td>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>10. Control → FANFT → control</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. FANFT</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>7</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a One of these was malignant with seeding of the pelvic peritoneal lining.

cant (p < 0.04 and < 0.006, respectively, for Groups 4 and 5). As in the rats fed FANFT and saccharin, none of the rats fed FANFT and tryptophan had normal bladders.

Unlike the small, solitary tumors observed in rats fed FANFT for 6 weeks followed by control diet, the tumors in rats fed FANFT followed by either tryptophan or saccharin were frequently multiple and usually large, filling the bladder lumen and distending the bladder. Areas of hyperplasia were generally present in other parts of the bladder mucosa not involved by tumor. The extent of invasion of the bladder lesions in the various groups is shown in Table 3, but invasion did not always correlate with size of lesions.

Several of the larger tumors had areas of necrosis, and occasionally calcification was present in these necrotic areas (Table 2). Calculi were not observed macroscopically in any of the groups of rats, but occasional microscopic calculi were observed trapped between papillary fronds of a tumor. Such calculi were observed in 2 rats in each of Groups 1 and 10 and in 3 rats in Group 11.

Scanning electron microscopy performed at Weeks 6, 12, and 104 of the experiment showed normal bladder mucosal surfaces in the rats in the control group and in the groups
Promotion in Bladder Carcinogenesis

The urinary bladders examined by scanning electron microscopy were also examined by transmission electron microscopy. Changes observed by transmission electron microscopy were similar to those described previously (33, 34) and were consistent with the types of lesions observed by light and scanning electron microscopy. There was gradual loss of asymmetrical membrane and fusiform vesicles as the lesions progressed. No asymmetrical membrane was present in microvilli.

One of the bladder tumors from Group 2 was transplanted s.c. to weanling male Fischer rats and has been successfully carried through 5 transplant generations. The histology of the original tumor showed both a transitional cell carcinoma and a highly anaplastic sarcoma invading through the bladder wall. Only the sarcoma grew in the transplanted tumors, and examination by transmission electron microscopy demonstrated plump tumor cells with large round to oval nuclei containing clumped chromatin and occasionally bizarre nucleoli. The cytoplasm contained large mitochondria with tubular cristae, well-developed Golgi caveolae, and abundant polyribosomes and endoplasmic reticulum filled with flocculent material. Occasional stereocilia were present. The cells lacked desmosomes and tight junctions, but some gap junctions were observed. Basement membrane-like material and collagen were produced. Myofilaments were not present. These features are consistent with a very poorly differentiated fibrosarcoma.

The most common non-bladder tumor was the interstitial cell tumor of the testis (Table 2), which frequently occurred bilaterally and appeared more often as the rats increased in age. Thus, few interstitial cell tumors were seen in the rats fed FANFT for long periods since none survived beyond Week 80 of the experiment. No correlation was observed between the occurrence of testicular tumors and the induction of bladder tumors. Testicular tumors are very commonly found spontaneously in older male control rats (22).

Other tumors observed in this experiment are summarized in Table 2 and also occurred with greater frequency with increasing age of the rats. Endocrine tumors were listed as adenomas without attempting to differentiate between hyperplastic nodules and adenomas. In addition to

One of the 2 rats fed FANFT for 6 weeks and examined at that time had small foci of hyperplastic epithelium with the luminal surface having numerous short, uniform microvilli and microridges, but a few pleomorphic microvilli were also present (Fig. 2). The other rat in this group at 6 weeks had only uniform microvilli with mild hyperplasia. The rats fed FANFT 6 weeks followed by control diet for 6 weeks and then examined showed normal mucosa by light and scanning electron microscopy, similar to the appearance previously described (33, 34). The rats fed FANFT 6 weeks followed by tryptophan for 6 weeks and then examined showed marked epithelial hyperplasia with nodular and papillary formation by light microscopy, and a nearly normal mucosa was observed by scanning electron microscopy with the surface being flat (Fig. 3). The epithelial cells were polygonal with microridges on the luminal surface, but a few foci were observed with some variation in cell size. Rats fed FANFT for 12 weeks and then examined showed marked epithelial hyperplasia with nodular and papillary formation by light microscopy with numerous pleomorphic microvilli present on the luminal cell surfaces when examined by scanning electron microscopy, similar to the appearance previously described (33, 34). The rats fed FANFT for 6 weeks followed by saccharin for 6 weeks and then examined had moderate epithelial hyperplasia (Fig. 4) with marked variation in cell size and shape. Cell surfaces were covered with numerous uniform microvilli, and some cells were covered with both numerous and uniform pleomorphic microvilli (Fig. 5). Rats fed FANFT for 6 weeks followed by tryptophan for 6 weeks and then examined were nearly normal by light and scanning electron microscopy, but greater variation in cell size (Fig. 6), and more foci with variations in size were present than in the rats fed FANFT for 6 weeks followed by control diet. Scanning electron microscopy of tumors from the various groups was similar to that previously described for urinary bladder tumors in rats (33, 34) with the surfaces having numerous pleomorphic microvilli (Fig. 7) as well as numerous uniform microvilli and ropy microridges. The one rat in Group 1 that did not have a bladder carcinoma had marked nodular hyperplasia, and the luminal surface had numerous pleomorphic microvilli (Fig. 8).

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Papillomas</th>
<th>Total</th>
<th>Non-Invasive</th>
<th>Stalk</th>
<th>Submucosa</th>
<th>Muscle</th>
<th>Serosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. FANFT → saccharin</td>
<td>19</td>
<td>0</td>
<td>18</td>
<td>11$^a$</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2. FANFT → control → saccharin</td>
<td>18</td>
<td>0</td>
<td>13</td>
<td>10$^a$</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1$^b$</td>
</tr>
<tr>
<td>3. FANFT → control → tryptophan</td>
<td>19</td>
<td>1</td>
<td>10</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4. FANFT → control → control</td>
<td>20</td>
<td>4</td>
<td>10</td>
<td>8$^a$</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5. FANFT → control</td>
<td>20</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6. FANFT → control</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td>2$^a$</td>
<td>6$^c$</td>
<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>7. Control → control</td>
<td>19</td>
<td>0</td>
<td>19</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>8. FANFT</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>6$^a$</td>
<td>1</td>
<td>8</td>
<td>17</td>
<td>8$^d$</td>
</tr>
</tbody>
</table>

$^a$ Sarcoma also present in one rat bladder.
$^b$ Metastases present.
$^c$ Sarcoma also present in 2 rat bladders.
$^d$ Metastases present in 2 rats.
the tumors listed in Table 2, most of which originated in various endocrine organs or soft tissues, the older rats occasionally showed mild, focal bile duct hyperplasia in the liver and mild, focal interstitial nephritis. All of these non-bladder lesions are common findings in older rats (22). In addition, a single rat in each of Groups 8 and 10 had hyperplastic nodules in the liver which are occasionally seen in older rats spontaneously (22).

**DISCUSSION**

The 2-stage process of carcinogenesis, initiation and promotion, was initially proposed and demonstrated in the murine skin cancer model (8, 9). Several properties of initiation and promotion have been characterized experimentally in that system (10, 67). Initiation by itself does not result in cancer but requires the action of a promoting agent to produce a cancer. Initiation is irreversible; application of the promoting substance can be delayed for long periods of time, and the carcinogenic process will still appear. Application of the promoting substance without initiation theoretically will not induce tumors, but long-term administration of promoting substances has usually resulted in low incidences of tumors (11, 12, 52, 65). Application of the promoter for periods adequate for promotion, but administered before the initiator, does not result in tumors. Although theoretically a pure initiator could exist, in practice subcarcinogenic doses of complete carcinogens have been used as initiating agents. In addition, it appears that the mechanisms of initiation and promotion differ biochemically (67). Most initiating agents are also mutagenic in various short-term *in vitro* assays, whereas promoting agents generally are not mutagenic or have only weak mutagenic activity. Promoting agents usually have the ability to induce hyperplasia of the target organ without initiation, but this does not progress to cancer unless the tissue has been initiated.

Although multistage carcinogenesis has been studied primarily in the murine skin cancer model, other organs recently have also been demonstrated to undergo a similar process. Phorbol administered systemically rather than topically has had weak promoting activity for skin carcinogenesis in mice (6), but it had greater promoting activity for liver and lung tumors in mice following initiation with dimethylnitrosamine (4) and for the breast in rats following initiation with 7,12-dimethylbenz(a)anthracene (5). Initiation and promotion involving hepatic carcinogenesis has also been reported, with a variety of chemicals used as initiating and promoting agents (21, 47). For the urinary bladder, the 2-stage process has been demonstrated in rats with a single intravesical instillation of MNU followed by either sodium saccharin or sodium cyclamate (29, 31, 32). The single instillation of MNU did not induce tumors, but saccharin or cyclamate alone given p.o. resulted in a very low incidence of bladder tumors after 2 years. Cyclophosphamide injected i.p. did not demonstrate promoting activity in the MNU experimental model.

Six weeks of 0.2% FANFT was chosen in the present experiment as the initiating agent because previous experiments in our laboratories had demonstrated that, although rats fed 0.2% FANFT for 6 weeks developed mild bladder epithelial hyperplasia, this had regressed when the rats were then fed the control diet so that the epithelium was normal at 52 (33) and 84 (34) weeks when examined by light and electron microscopy. The appearance of tumors in the rats fed 0.2% FANFT for 6 weeks followed by control diet in the present experiment was unexpected in the light of these previous studies. To our knowledge, neither the carcinogen nor our laboratory procedures had changed except that the present experiment was continued to a total of 104 weeks. In previous experiments (27, 33, 34), examination of the urinary bladders by scanning electron microscopy showed the mucosal surface to have uniform microvilli but not pleomorphic microvilli after 6 weeks of 0.2% FANFT. In the present experiment, however, pleomorphic microvilli were present in 1 of 2 bladders examined at 6 weeks. This might indicate greater susceptibility of this group of rats to the carcinogen than in previous experiments, but male inbred Fischer weanling rats from the same supplier were used in both instances and under the same conditions. An alternative explanation is that, at 6 weeks, a small percentage of the rats fed 0.2% FANFT have pleomorphic microvilli, and by chance we observed some in one rat in this experiment and not in previous experiments. Since pleomorphic microvilli appear to be a marker of irreversibility in the FANFT-Fischer rat model (27, 33, 34), finding them in few rats after 6 weeks of FANFT is consistent with the low incidence of bladder tumors observed when these rats are then fed control diet until a total of 2 years as in the present experiment.

The results of this experiment and the studies of Hicks with MNU are consistent with the 2-stage theory of carcinogenesis for the urinary bladder. Features consistent with the theory and similar to the murine skin model are the sequential administration of 2 chemicals to induce cancer, the use of a low dose of a complete carcinogen as the initiating agent, and the lack of (or very low) carcinogenic activity of the promoting chemicals when administered without previous initiation. Previous experiments in rats with one-generation administration of saccharin or of cyclamate plus saccharin have resulted in very low incidences of bladder tumors (15, 16, 29, 31, 32, 43, 46, 49), and negative results have been observed in some species (23) including mice (37), hamsters (2), and a few other studies in rats (26, 53, 56, 61, 62). In the present experiment, the number of rats in each group might well have been insufficient to detect a low level of carcinogenic activity of saccharin as previously reported. Moreover, rats in the present experiment did not receive saccharin until after the sixth week of the experiment when they were 10 weeks old. In previous experiments evaluating saccharin, feeding of the chemical was begun at an earlier age. It should be noted, however, that our studies were done to study the possible promoting effect of saccharin in this model system, not the carcinogenic activity of saccharin. Two-generation studies, the administration of saccharin over 2 generations including during pregnancy and lactation, resulted in higher incidences of bladder tumors than did one-generation studies (7).

Two possible explanations can be proposed regarding the low incidence of bladder tumors when only saccharin is administered, whereas high incidences occur when sac-
charin follows either MNU or FANFT administration. The low incidence resulting with saccharin alone may reflect the background incidence of initiated cells in the urinary bladder occurring due to spontaneous changes, factors in the diet, or endogenous factors. Low levels of nitrosamines, for example, have been found in many grain diets and can also be formed endogenously from amines and nitrite (55, 59). An alternative explanation is that saccharin is a complete carcinogen but has only weak initiating capabilities. This would be similar to the results in skin carcinogenesis where long-term administration of promoting agents results in a low incidence of tumors (11, 12, 52, 65). Evaluation of saccharin as an initiating chemical in the mouse skin model showed it to have weak but not statistically significant activity (54). If saccharin is a complete carcinogen, then saccharin following either MNU or FANFT may represent a synergistic reaction resulting in a summation effect (44, 60, 64). At present, there are insufficient data available to state which of these alternatives are correct. Reversing the sequence of administration might provide relevant new information, but the long period of time required for the promoting phase for the urinary bladder will make that experiment difficult to design properly for adequate evaluation.

The 6-week delay between the end of FANFT administration and the beginning of either saccharin or tryptophan feeding, although relatively short, results in return of the bladder mucosa to an essentially normal appearance by light and electron microscopy (20, 33, 34). Also, FANFT is rapidly eliminated from the rat via the urine and feces (58), and little if any would be expected to be present after 6 weeks on control diet. Since both saccharin and tryptophan induced bladder tumors even after the 6-week delay after FANFT administration, the initiated changes were irreversible at least for that period of time as was the stage of initiation in the mouse skin model.

Other properties of saccharin are also consistent with its being at least a promoting agent. In general, saccharin lacks mutagenic activity in short-term in vitro assays (18, 24, 36, 40, 57, 66). The weak mutagenic activity occasionally found might be due to impurities present in the test preparations (24, 57). These impurities may be acting as initiators in vivo with the saccharin acting as the promoter. However, it seems highly unlikely that these impurities can account for all of the activity attributed to saccharin since they are present at very low levels. For the impurities to account for the carcinogenic activity of saccharin, they would have to be some of the most potent carcinogens ever discovered. Our material was examined for o- and p-toluene sulfonamide levels, which were very low (<0.03 ppm), but not for other impurities. Saccharin, in addition to its low or absent mutagenic activity, is metabolized little or not at all (18), and it does not bind to DNA (38).

Most promoting agents are able to induce hyperplasia of the target organ even without initiation (67). Hyperplasia of the bladder secondary to saccharin has generally not been reported, but sequential examination of the animals has not been performed. However, Hicks* has recently found that saccharin alone does induce bladder urothelial hyperplasia. The mechanism by which saccharin induces its promoting effect is unknown. An indirect effect by alteration of the urine is possible but would seem unlikely considering the results with tryptophan in the present experiment. Also, promoting activity in vivo was demonstrated in the mouse embryo cell culture assay system without the presence of urine, although relatively high levels of saccharin were required (42). It has been suggested that calculus formation is related to the saccharin effect, but in our studies we did not find calculus formation to be a prerequisite. The initial reports of the bladder carcinogenicity of saccharin involved the surgical placement of pellets containing saccharin into the mouse bladder (1, 15, 16). However, in our experiments and that with MNU, calculi were infrequently present in the bladders of rats receiving saccharin and developing tumors.

and when present the calcified material was within necrotic areas of the tumor and not in the lumen of the bladder.

These areas of calcification are most probably related to the presence of tumor tissue and tumor necrosis rather than preceding tumor formation.

The tumors produced by saccharin after FANFT feeding were malignant, and several of them were invasive. In addition, the tumors persisted after the chemical was discontinued in the diet at the end of Week 83 of the experiment, 21 weeks before the end of the experiment.

DL-Tryptophan also demonstrated promoting activity in the present experiment although it was not as potent as saccharin. This may be due to the difference in dose as well as to other factors. Many of the features characterizing saccharin as a promoting agent are the same for tryptophan. A 6-week delay after FANFT feeding did not decrease its activity as a promoter, and tryptophan alone did not induce tumors. Hyperplasia due to tryptophan was seldom observed in the present experiment, but proliferation has been reported in rats (41) and dogs (50). Also, tryptophan and its metabolites lack mutagenic activity in in vitro assays (13). In the present experiment, DL-tryptophan was fed rather than only the biologically important L-tryptophan. Promoting activity by DL-tryptophan has been suggested to occur also in the dog (51), and weak promoting activity has been reported for L-tryptophan in mice following FANFT initiation (39).

Since the report of Dunning et al. (25) showing increased bladder carcinogenicity of 2-acetylaminofluorene when coadministered with tryptophan, a relationship has been sought between tryptophan, its metabolites, and bladder carcinogenesis (14, 48). Several of the metabolites are increased quantitatively in the urine of some bladder cancer patients, and some of the metabolites are bladder carcinogens using the pellet implantation technique (14, 48). However, tryptophan administered to experimental animals p.o. did not induce bladder cancer (14, 41, 45, 51). The present experiment indicates that tryptophan might not be a complete bladder carcinogen but rather is a promoting agent for the bladder that may be relevant to bladder carcinogenesis in some human cases. One would expect that if this were true then bladder cancer patients with abnormal tryptophan metabolite levels would have an increased rate of recurrence of their bladder tumors following therapy since they continue to have a promoter present in their urine. Such a result has been reported in a small series of patients by Yoshida et al. (68). If the abnormal metabolite

* R. M. Hicks, personal communication.
level could be eliminated, e.g., by administering vitamin B6, recurrences should be reduced or prevented. Such a result has recently been reported by Byar and the Veterans Administration Cooperative Urological Research Group (17), although urinary tryptophan metabolite levels were not measured. The importance of tryptophan metabolites in the etiology of bladder cancer and the usefulness of vitamin B6 in the treatment of bladder cancer, particularly low-grade superficial papillary tumors, need further evaluation.

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Fig. 1. Luminal surface of the bladder of a rat fed saccharin (Group 3) and sacrificed at the end of the experiment (2 years). The typical microridge appearance seen in normal rat bladder is present along with an occasional bleb. × 6,000.

Fig. 2. Pleomorphic microvilli present on the bladder luminal surface of a rat fed FANFT for 6 weeks and then killed. × 10,000.

Fig. 3. The luminal surface of the bladder of a rat fed FANFT for 6 weeks followed by control diet for 6 weeks and then killed. The bladder mucosa has returned to near normal with a flat surface composed of large polygonal cells with microridges. Occasional foci show some variation in cell size. × 500.

Fig. 4. Hyperplastic bladder epithelium from a rat fed FANFT for 6 weeks followed by saccharin for 6 weeks and then killed. × 200.
Fig. 5. Higher magnification of the bladder shown in Fig. 4 showing pleomorphic microvilli on the surface in addition to the short, uniform microvilli. x 4,000.

Fig. 6. The bladder surface of a rat fed FANFT for 6 weeks followed by tryptophan for 6 weeks is near normal, similar to that shown in Fig. 3, but foci with variations in cell size are more numerous. x 200.

Fig. 7. Transitional cell carcinoma in a rat fed FANFT for 6 weeks and then fed saccharin. The rat was killed at the end of the experiment. x 200.

Fig. 8. Numerous pleomorphic microvilli present on the bladder surface of a rat fed FANFT for 6 weeks followed by saccharin and killed at the end of the experiment. This was the only rat in Group 1 that did not have a bladder carcinoma. x 8,600.
Promoting Effect of Saccharin and dl-Tryptophan in Urinary Bladder Carcinogenesis


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