Acute Changes in Nucleic Acid and Protein Synthesis in the Mouse Bladder Epithelium Induced by Three Bladder Carcinogens

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SUMMARY

The effect of a single oral dose of 4-ethylsulfonylnaphthalene-1-sulfonamide, 2-acetylaminofluorene, and 3-aminodibenzofuran on DNA, RNA, and protein synthesis in the urinary bladder and liver of the mouse has been investigated. Each chemical stimulated RNA and DNA synthesis in the bladder, but the magnitude and timing of the response was different in each case. Administration of 4-ethylsulfonylnaphthalene-1-sulfonamide in the diet produced an increase in DNA synthesis in the mouse bladder which lasted for 4 weeks, whereas with 2-acetylaminofluorene, increased DNA synthesis occurred only during the first week. The effect of 4-ethylsulfonylnaphthalene-1-sulfonamide on nucleic acid synthesis was specific for the bladder, whereas 2-acetylaminofluorene stimulated both RNA and DNA synthesis in the liver and 3-aminodibenzofuran induced a small increase in DNA synthesis in male, but not in female, mouse liver.

INTRODUCTION

The mouse bladder epithelium normally exhibits a low level of DNA synthesis and a low mitotic index (0.01%) (10, 14, 21, 22) and is an example of a quiescent cell population which may be stimulated into active DNA synthesis and cell division (3). This may be achieved by a variety of means, including physical wounding (26), implantation of a pellet into the lumen of the bladder (10), or chemical injury by bladder carcinogens (11, 20, 22).

The present work is a biochemical study of the kinetics of the stimulation of DNA and RNA synthesis in the mouse bladder and complements a parallel investigation with autoradiographic and quantitative cytochemical techniques (22). Three carcinogens were used: ENS, ABF, and AAF, all of which induce hyperplasia of the mouse bladder epithelium when fed for 1 to 8 weeks (9) and bladder tumors on prolonged feeding (7, 8, 28). ENS was used most extensively in this study as in earlier biological studies of chemically induced proliferation in mouse bladder, and the actions of AAF and ABF were studied for comparison and to determine whether other bladder carcinogens had a similar action. Some of the present results have previously been reported in abstract (20).

MATERIALS AND METHODS

Chemicals. ENS was prepared by the method of Brimelow and Vasey (4); ABF was prepared by the reduction of 3-nitrodibenzofuran with hydrogen (2.5 atmospheres) in the presence of palladium (5%) on charcoal; and AAF was obtained from Koch-Light, Ltd., Colnebrook, England. Actinomycin D was obtained from Merck, Sharp and Dohme, Inc., West Point, Pa.

Radiochemicals. Thymidine-6-3H (5.0 Ci/mmole), uridine-5-3H (5.0 Ci/mmole), leucine-4,5-3H (10.3 Ci/mmole), and 125IUDR (35.7 µCi/mg) were obtained from the Radiochemical Centre, Amersham, England.

Conduct of Animal Experiments. ENS (5 and 40 mg/ml) and ABF (10 mg/ml) were administered by stomach tube in propylene-1,2-diol:water:gum tragacanth (1:13:6, v/v) (22). AAF (40 mg/ml) was given similarly in corn oil. ENS and AAF were incorporated in the diet as previously described (28). Thymidine-3H (0.25 µCi/g), uridine-3H (0.25 µCi/g), and leucine-3H (0.4 µCi/g) were injected i.p., the thymidine and uridine 1 hr before death and the leucine 30 min before death. Animals given 125IUDR were fed NaI (0.1%) in drinking water for 3 days prior to and during the experiment to lower the incorporation of free 125I into tissue constituents. 5-Fluoro-2-deoxyuridine (10-7 mole, i.p.), an inhibitor of DNA synthesis (16) was given 1 hr before 125IUDR in an attempt to enhance the incorporation of IUDR into DNA. Animals were killed 16 hr after injection of 125IUDR to allow free 125I to be excreted. 125IUDR is available for incorporation for approximately 1 hr (18). The radioactivity was determined in the whole tissue.

Extraction of Nucleic Acids and Protein

RNA. RNA was extracted by the method of Alston and Thomson (1). The tissues were homogenized for 10 sec at...
6000 rpm in a Silverson homogenizer (Silverson Machines, Ltd., Chesham, England) in ice-cold 0.05 M Tris-HCl buffer, pH 7.3, containing 0.1 M NaCl and 0.001 M EDTA (disodium salt) (24).

DNA. Individual halved bladders were partially disintegrated ultrasonically (Kerry's Ultrasonics, Ltd., Basildon, Ltd., Chesham, England) in 2.0 ml ice-cold saline-citrate (0.15 M NaCl-0.015 M disodium citrate) for 15 sec at 18 to 22 kilocycles/sec (ultrasonic probe size, 6.1 cm long x 0.9 cm diameter). A homogenate (epithelial fraction) was produced and a husk of tissue, muscle, and connective tissue was left (nonepithelial fraction). The isolation of DNA from these fractions involved extraction with chloroform:butanol (3:1, v/v), producing a 2-phase system, the formation of which was accelerated by centrifugation at 4000 rpm. The epithelial fraction comprised the upper layer and was separated from the lower layer with a Pasteur pipet with a bent tip. The husk (nonepithelial fraction) remained at the interface and was removed with a pair of fine forceps. It was washed with saline-citrate (2 ml) which was added to the epithelial fraction. The husk was subsequently homogenized in 2.0 ml saline-citrate in the Silverson homogenizer at 6000 rpm for 10 sec. About 40% of the hydroxyproline of the bladder was in the epithelial fraction, indicating that this fraction contained some of the connective tissue which lies between the muscle and the epithelium. Hydroxyproline was estimated by the method of Woessner (27). DNA was extracted from both fractions of the bladder by the method of Colburn and Boutwell, omitting the treatment with ribonuclease (12). About 8 to 12 μg of DNA were extracted from the epithelial fraction of each bladder. DNA was extracted from the liver by the Schmidt-Thannhauser procedure (25).

Protein. Protein was extracted from the epithelial fraction of the bladder by precipitation with 5 volumes of methanol. The precipitate was washed with chloroform:methanol (3:1, v/v) to remove lipid and heated with N perchloric acid at 70°C for 1 hr to remove nucleic acids.

Determination of Specific Activity

DNA. Bladder DNA was dissolved in 0.2 ml of water, applied to discs of glass-fiber paper (Whatman GF 83; 2.1-cm diameter), and dried. Radioactivity was measured in 2.0 ml of scintillation fluid/vial; subsequently, the discs were dried and extracted with 2.5 ml N perchloric acid at 70°C for 1 hr. The extracts were cooled and deoxyribose was determined by the diphenylamine method (6). The specific activity was expressed as cpm/μg deoxyribose.

Liver DNA was obtained as a perchloric acid extract; an aliquot (0.1 ml) was assayed for radioactivity in 10 ml scintillation fluid after solubilization with ethanol:Hyamine 10X (Nuclear Enterprise, Ltd., Edinburgh, Scotland) (2:1, v/v). Deoxyribose was determined as above. The specific activity was expressed as dpm/μg deoxyribose.

RNA. Bladder and liver RNA were obtained as perchloric acid extracts. An aliquot (0.1 ml) was assayed for radioactivity as above, and the ribose content of the remainder was determined by the orcinol method (23). The specific activity was expressed as dpm/μg ribose.

Protein. The protein precipitate was dissolved in 0.4 ml N NaOH. The radioactivity was determined on 0.2 ml, the remaining 0.2 ml being used to determine the protein content by the biuret method, with bovine serum albumin as the standard (15). The specific activity was expressed as dpm/μg protein.

RESULTS

Incorporation of Thymidine-3H into Bladder DNA. The incorporation of thymidine-3H into DNA from the epithelial fraction of the bladder of female A × IF mice is shown in Table 1. There is a statistically significant difference between the levels of incorporation at 20 and 40 min (0.01 > p > 0.001) and at 40 and 60 min (p < 0.001), but not at 60 and 80 min (p > 0.05), probabilities being calculated by the t test. In all subsequent experiments in which DNA synthesis was measured, the animals were killed 1 hr after the injection of thymidine-3H.

<table>
<thead>
<tr>
<th>Time after i.p. injection of thymidine-3H (min)</th>
<th>Specific activity of DNA (cpm/μg deoxyribose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>40</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>60</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>80</td>
<td>35 ± 5</td>
</tr>
</tbody>
</table>

aThe values are mean ± S.D. and were derived from 16 animals.

Effect of ENS on the Mouse Bladder. The incorporation of 125IUDR into the whole bladder of A × IF mice is shown in Chart 1. As earlier autoradiographic studies had shown that ENS-induced DNA synthesis occurred mainly in the epithelial cells (22), a method of partial ultrasonic disintegration was devised whereby a fraction of the bladder was obtained which contained the epithelial cells and part of the connective tissue as judged by the hydroxyproline content. DNA synthesis was examined in later experiments by measuring the incorporation of thymidine-3H into DNA because of the limited availability of the gamma counter. The increase in DNA synthesis produced by ENS was chiefly in the epithelial fraction, whereas the stimulation of the nonepithelial fraction was less pronounced and occurred later (Chart 2). All subsequent determinations of DNA synthesis in the bladder were made on the epithelial fraction. ENS had a similar effect in male A × IF mice, but larger doses than those used in female mice were necessary (Chart 3). ENS (0.5 mg) did not affect DNA synthesis in the liver, skin, kidney, spleen, thymus, bone marrow, or duodenum of female A × IF mice. The vehicle alone was without effect on DNA synthesis in the bladder and in all subsequent experiments a group of otherwise untreated mice was given the radioactive precursor as a control. The administration of
Chart 1. Incorporation of $^{125}$IUDR into bladder DNA of female A X IF mice given a single dose p.o. of ENS (0.5 mg). This is considered to be 0 time. $^{125}$IUDR (6 mCi/mg; 5 µCi) was administered by i.p. injection at 6, 12, 24, 36, 48, and 72 hr after the ENS, and the animals were killed 16 hr later to allow for the excretion of free iodine. The values of specific activity are plotted against the times at which $^{125}$IUDR was given. Points, mean of 8 animals; vertical lines, 1 S.D.

5-fluoro-2-deoxyuridine 1 hr before the administration of thymidine-$^3$H did not reduce the large variations between individual mice and was not used in further experiments.

RNA synthesis was examined by measuring the incorporation of uridine-$^3$H into RNA from the whole bladder inasmuch as RNA could not be extracted from the epithelial fraction produced by ultrasonic homogenization. After a single dose of ENS (0.5 mg) to female A X IF mice, there was a decrease in RNA synthesis at 5 hr to about 40% of the control value. The peak of synthesis occurred at 16 hr (Chart 4). The stimulation of protein synthesis in the epithelial fraction could not be detected until about 20 hr after administration of ENS (Chart 4).

Because the stimulation of cell division in the bladder epithelium may be essential before chemical carcinogens can induce tumors (10), it was pertinent to examine the action of ENS on bladders already stimulated into active nucleic acid synthesis by an earlier dose of the chemical. The effect of a 2nd dose of ENS (0.5 mg) given 24 hr after the 1st on DNA and RNA synthesis is shown in Chart 5. DNA synthesis decreased 6 hr after the 2nd dose and did not increase for about 12 hr. The peak of synthesis occurred about 20 hr after the 2nd dose. The peak of RNA synthesis in the whole bladder occurred about 12 hr after the 2nd dose of ENS and declined rapidly.

Effect of Actinomycin D on ENS-stimulated DNA and RNA Synthesis. The effect of actinomycin D on ENS-stimulated DNA synthesis in the epithelial fraction and RNA synthesis in the whole bladder of female A X IF mice is shown in Tables 2 and 3. Actinomycin D (3 µg) given 22 hr after ENS (0.5 mg) reduced the stimulated DNA synthesis at 30 hr by 50% (Table 2). A similar dose, given 10 hr after ENS, i.e., before the peak of RNA synthesis, reduced the stimulated DNA synthesis at 30 hr to the level in untreated control animals. Actinomycin D (3 µg) given 10 hr after ENS reduced RNA synthesis by 70% within 2 hr (Table 3).

Effect of AAF and ABF on Mouse Bladder. The effects of AAF (4 mg) and ABF (2 mg) on DNA synthesis in the epithelial fraction of the bladder are shown in Chart 6 and the effects on RNA synthesis in the whole bladder in Chart 7. AAF was examined in male IF mice (Charts 6a and 7a) and ABF was examined in male (Charts 6b and 7b) and female (Charts 6c and 7c) IF X C57 mice. A single dose of AAF reduced DNA synthesis, which did not begin to increase until 24 to 32 hr after dosing. The peak of DNA synthesis was reached between 42 and 54 hr, the normal level being regained by 72 hr (Chart 6a). A similar dose of AAF produced 3 waves of RNA synthesis in the whole bladder (Chart 7a).

ABF is a more potent bladder carcinogen in male than in female IF X C57 mice, and exerts different effects on nucleic acid synthesis in the bladders of the 2 sexes (8). In male IF X C57 mice, a single dose of ABF (2 mg) induced 2 waves of DNA synthesis in the epithelial fraction, 1 between
The effect of actinomycin D on ENS-stimulated DNA synthesis in the epithelial fraction of the bladder

ENS was given p.o. and actinomycin D was given by i.p. injection. The mice were killed 30 hr after the administration of ENS. Thymidine-³H (0.25 µCi/g) was given 1 hr before death. There were 10 animals in each group.

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Specific activity of DNA (cpm/µg deoxyribose)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35 ± 14</td>
</tr>
<tr>
<td>ENS (0.5 mg)</td>
<td>291 ± 42</td>
</tr>
<tr>
<td>ENS (0.5 mg) + actinomycin D (3 µg) at 22 hr</td>
<td>145 ± 44</td>
</tr>
<tr>
<td>ENS (0.5 mg) + actinomycin D (3 µg) at 10 hr</td>
<td>37 ± 17</td>
</tr>
<tr>
<td>ENS (0.5 mg) + actinomycin D (6 µg) at 10 hr</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>ENS (0.5 mg) + actinomycin D (12 µg) at 10 hr</td>
<td>12 ± 5</td>
</tr>
</tbody>
</table>

¹Mean ± S.D.

6 and 16 hr and a 2nd broader wave between 48 and 72 hr (Chart 6b). ABF also induced 2 waves of RNA synthesis in the whole bladder of male IF X C57 mice, the 1st within the first 12 hr and the 2nd commencing about 36 hr after dosing (Chart 7b). In female IF X C57 mice, ABF caused 2 waves of DNA synthesis; both were broad and occurred later than the 2 waves in the bladder of males (Chart 6c). ABF initially depressed RNA synthesis in whole bladder of female IF X C57 mice, after which there was a period of increased RNA synthesis (Chart 7c).

Effect of AAF and ABF on the Liver. AAF and ABF are liver carcinogens in the strains of mouse used in these experiments (2, 8). AAF produced 1 peak of DNA synthesis between 48 and 72 hr (Chart 8a). ABF produced a small increase in DNA synthesis in the liver of male IF X C57 mice (Chart 8b), but it was without effect in the liver of female IF X C57 mice (Chart 8c).
DISCUSSION

There was considerable variation in the level of incorporation of radioactive precursors into bladder DNA, RNA, and protein of individual mice. The standard deviation of thymidine-3H-labeled DNA from the epithelial fraction of the bladder of otherwise untreated mice was 36% of the mean, whereas that from the duodenum was only 18%. The

**Table 4**

<table>
<thead>
<tr>
<th>Duration (wk)</th>
<th>0.005% ENS in A X IF (F)</th>
<th>0.05% AAF in IF (M)</th>
<th>0.05% AAF in IF (F)</th>
<th>0.05% AAF in IF (M)</th>
<th>0.05% AAF in IF (F)</th>
<th>0.05% AAF in IF (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37 ± 17 (30)©</td>
<td>35 ± 5 (10)</td>
<td>45 ± 7 (10)</td>
<td>267 ± 31 (10)</td>
<td>310 ± 26 (10)</td>
<td>370 ± 31 (10)</td>
</tr>
<tr>
<td>1</td>
<td>57 ± 24 (10)</td>
<td>55 ± 27 (10)</td>
<td>65 ± 35 (10)</td>
<td>255 ± 24 (10)</td>
<td>290 ± 22 (10)</td>
<td>345 ± 24 (10)</td>
</tr>
<tr>
<td>2</td>
<td>119 ± 100 (18)</td>
<td>25 ± 6 (10)</td>
<td>31 ± 5 (10)</td>
<td>215 ± 38 (10)</td>
<td>210 ± 34 (10)</td>
<td>250 ± 38 (10)</td>
</tr>
<tr>
<td>3</td>
<td>68 ± 25 (18)</td>
<td>25 ± 9 (10)</td>
<td>32 ± 7 (10)</td>
<td>215 ± 43 (10)</td>
<td>280 ± 55 (10)</td>
<td>315 ± 43 (10)</td>
</tr>
<tr>
<td>4</td>
<td>94 ± 33 (18)</td>
<td>36 ± 16 (10)</td>
<td>42 ± 18 (10)</td>
<td>240 ± 34 (10)</td>
<td>435 ± 65 (10)</td>
<td>470 ± 34 (10)</td>
</tr>
<tr>
<td>8</td>
<td>25 ± 9 (10)</td>
<td>35 ± 18 (10)</td>
<td></td>
<td>210 ± 39 (10)</td>
<td>210 ± 31 (10)</td>
<td></td>
</tr>
</tbody>
</table>

©Number of animals used for each determination is shown in parentheses.
Carcinogens and Bladder Nucleic Acid Synthesis

Chart 7. Incorporation of uridine-3H (0.25 μCi/g) into RNA from the whole bladder. a, male IF mice given a single dose p.o. of AAF (4 mg). b, male IF X C57 mice given a single dose p.o. of ABF (2 mg). c, female IF X C57 mice given a single dose p.o. of ABF (2 mg). Uridine-3H was given 1 hr before death. Points, mean of 12 animals; vertical lines, 1 S.D.

Chart 8. Incorporation of thymidine-3H (0.25 μCi/g) into liver DNA. a, male IF mice given a single dose p.o. of AAF (4 mg); b, male IF X C57 mice given a single dose p.o. of ABF (2 mg); c, female IF X C57 mice given a single dose p.o. of ABF (2 mg). Thymidine-3H was given 1 hr before death. Points, mean of 12 animals; vertical lines, 1 S.D.

variation is believed to reflect the behavior of bladder tissue, rather than inconsistencies in techniques. Comparable variation has been observed in other studies of bladder epithelial proliferation involving autoradiography (22) and in the present study of the incorporation of [125I]UDR into bladder DNA. In neither instance was DNA extracted from the tissue.

The specific activity of “1-hr-labeled” RNA from the bladder of untreated animals varied to a greater extent (30%) than duodenal (18%), liver (19%), and kidney (19%) RNA. This variation may be due partly to changes in the hormonal or nutritional status of the animals. The specific activity of bladder 1-hr-labeled RNA from untreated male mice was lower and less variable (1000 ± 280 dpm/μg ribose) than that from untreated female mice (1528 ± 650 dpm/μg ribose) (0.01 > p > 0.001, by t test) (T. A. Lawson and K. M. Dawson, unpublished results). The larger variation in the female may result from mutual disturbances of the estrous cycle caused by the grouping together of female mice (19).

The stimulation of DNA synthesis in the epithelial fraction and of RNA synthesis in the whole bladder are sensitive to actinomycin D. The greater inhibitory action on stimulated RNA synthesis of actinomycin D given before rather than after the peak of RNA synthesis indicates that part, at least, of the stimulated RNA synthesis in the whole bladder is directly involved in the preparation for epithelial DNA synthesis. In an attempt to overcome the difficulty in comparing DNA synthesis in the epithelial fraction with RNA synthesis in the whole bladder, a technique for the separation of an epithelial fraction by mechanical abrasion has recently been developed. It has been found that, after the administration of ENS, both RNA and DNA synthesis occur to a considerably greater extent in this fraction than in the residual tissue (T. A. Lawson, unpublished results).

The stimulation of DNA synthesis may be due to the direct action of the carcinogen or a metabolite on that part of the genome which controls DNA synthesis, or it may result indirectly from changes in the integrity of individual cells or the whole tissue. Examples of both mechanisms have been described in other in vivo systems (3). The earliest change detected in the study of the action of ENS was a decrease in 1-hr-labeled RNA synthesis in the whole bladder, followed at about 6 hr by an increase to a maximum at about 16 hr. ENS causes gross damage to the surface cells of the bladder epithelium, producing intense vacuolation and autophagocytosis, as evidenced by the marked increase of lysosome formation by 8 hr (22). Hodgson (17), using a scanning electron microscope, found evidence of superficial cell desquamation at 24 hr.

A 2nd dose of ENS, given 24 hr after the 1st, leads to an apparent inhibition of DNA synthesis in the epithelial fraction. This may be due to a direct action of the chemical on the tissue or to a change in the availability of precursors, which it has been suggested may be responsible for similar alterations in RNA synthesis in other systems (5).
From the results with single doses of ENS, ABF, and AAF, it is concluded that the increased DNA synthesis is probably a response to chemically induced cellular injury. AAF, which induces the smallest total increase in thymidine-3H incorporation, induces less damage than the other chemicals (M. Wood, personal communication).

The changes in nucleic acid synthesis induced by the 3 carcinogens in the bladder cannot be compared quantitatively, because different strains of mouse and different doses were used in order to relate the present work to earlier studies. These compounds probably require metabolic alteration for carcinogenic activity, and the administration of equimolar amounts would not ensure that equivalent urinary concentrations of the active metabolites were attained.

The experiments in which AAF and ENS were given in the diet show that the continuous feeding of AAF does not lead to a sustained high level of DNA synthetic activity in the bladder. With ENS there is a decrease in the number of mice which respond to the stimulus as the feeding continues (13). The maintenance of a high level of DNA synthesis does not therefore appear to be necessary in bladder carcinogenesis.

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