The effect of various carcinogenic and non-carcinogenic substances on the development of urinary bladder tumors in rats induced by N-butyl-N-(4-hydroxybutyl)nitrosoamine was examined histologically.

Pre- or post-treatment with N-nitrosopiperidine, N-nitrosomorpholine, diethylnitrosamine, or N-2-fluorenylacetamide inhibited the incidence of urinary bladder tumors in rats induced by N-butyl-N-(4-hydroxybutyl)nitrosoamine, but the production of hyperplasias and papillomas in the urinary bladder epithelium was not inhibited by pre- or post-treatment with several carcinogens. On pretreatment with various hepatotoxic and nephrotoxic substances, 4-chloroacetanilide and 1-naphthyl isothiocyanate were found to inhibit the production of urinary bladder cancer induced by N-butyl-N-(4-hydroxybutyl)nitrosoamine, the latter especially inhibiting not only the development of cancer but also hyperplasia and papilloma of the urinary bladder epithelium in rats induced by this carcinogen.

When the two carcinogens, N-butyl-N-(4-hydroxybutyl)nitrosoamine and N-2-fluorenylacetamide, were administered together, their action of inducing urinary bladder cancer was synergistic. Moreover, DL-tryptophan promoted their actions in producing urinary bladder tumors in rats.

The selective carcinogenic action of N-butyl-N-(4-hydroxybutyl)nitrosoamine on the urinary bladder of rats, mice, and dogs has been reported by many investigators. Histological and electron microscopic findings in vivo and in vitro on urinary bladder tumorigenesis in rats induced by N-butyl-N-(4-hydroxybutyl)nitrosoamine have also been reported. The metabolic pathways and carcinogenic activities of several derivatives of this carcinogen have been studied by Okada et al.

The present paper reports the effect of various carcinogenic substances, such as dimethyl-nitrosoamine, diethylnitrosoamine, N-nitrosopiperidine, N-nitrosomorpholine, and N-2-fluorenylacetamide, and non-carcinogenic chemicals, such as 4-chloroacetanilide, 1-naphthyl isothiocyanate, N-(3,5-dichlorophenyl)succinimide, and DL-tryptophan, on urinary bladder tumorigenesis in rats induced by N-butyl-N-(4-hydroxybutyl)nitrosoamine. The synergistic or antagonistic action between carcinogenic or non-carcinogenic substances and N-butyl-N-(4-hydroxybutyl)nitrosoamine in urinary bladder tumorigenesis in rats was also examined.

Materials and Methods

Male Wistar rats (Fuji Animal Farm, Tokyo), weighing an average of 172.9 g, were used. The chemicals, N-butyl-N-(4-hydroxybutyl)nitrosoamine (Izumi Chemical Co., Yokohama), 1-naphthyl isothiocyanate (Tokyo Kasei Co., Tokyo), 4-chloroacetanilide (Nakarai Chemical Ltd., Kyoto), DL-tryptophan (Yoneyama Chemical Co., Osaka), N-(3,5-dichlorophenyl)succinimide (Sumitomo Chemical Co., Osaka), dimethyl- and diethyl-nitrosoamine (Tokyo Kasei Co., Tokyo), N-nitrosopiperidine and N-nitrosomorpholine (K & K Laboratories Inc., U.S.A.), and N-2-fluorenylacetamide (Kodak Eastman Organic Chemicals, U.S.A.) were given in various amounts in the drinking water, in commercial stock diet (Oriental Yeast Co., Tokyo), or in the basal diet described previously.

Experimental Series I A total of 254 male rats were used in this series and were divided into 18 groups. Rats were given water with or without 0.05% N-butyl-N-(4-hydroxybutyl)nitrosoamine for 8 weeks. For 4 weeks before or after treatment with the carcinogen, the animals were fed on basal diet containing 0.03%
N-nitrosopiperidine or 0.025% N-2-fluorenylacetamide, and were given 0.015% N-nitrosomorpholine or 0.005% diethylnitrosoamine in their drinking water. After administration of the carcinogens, animals were fed on stock diet for 8 weeks and then killed by ether for histological observations. The schedules of administration of carcinogenic substances in each group are given in Fig. 1.

**Experimental Series II** A total of 155 male rats were used in this series and were divided into 9 groups. Animals were given water with or without 0.025% N-butyl-N-(4-hydroxybutyl)nitrosoamine for 8 weeks. Before treatment with the carcinogen, the animals were fed on basal diet containing nephrotoxic substances such as 0.25% 4-chloroacetanilide or 0.5% N-(3,5-dichlorophenyl)succinimide for 8 weeks, or on the basal diet containing hepatotoxic substances such as 0.06% 1-naphthyl isothiocyanate for 8 weeks or 0.01% dimethylnitrosoamine for 4 weeks. The schedules of administration of chemicals in each group are given in Fig. 2. All the rats were killed by ether for histological studies 20 weeks after the start of administration of N-butyl-N-(4-hydroxybutyl)nitrosoamine.
Experimental Series III  A total of 116 male rats were used in this series and were divided into 8 groups. Rats were given drinking water containing 0.001% N-butyl-N-(4-hydroxybutyl)nitrosoamine for 40 weeks and, at the same time, the animals were also fed on basal diet containing 0.005% N-2-fluorenylacetamide or 1.5% DL-tryptophan for 40 weeks. Control animals were given basal diet alone. The experimental design for each group is shown in Fig. 3. All the rats were killed by ether for histological studies 40 weeks after the start of the experiments.

The animals were housed in wire cages in an air-conditioned room at 24°C and weighed weekly. Animals which died before the time of sacrifice were excluded.

Histopathological Observations  The liver and both kidneys were weighed and samples of these organs were taken for histological observation. The urinary bladder was punctured at the urethra, and 0.5 ml of 10% buffered formaldehyde solution was injected into the bladder. Then tissues were fixed and stained with Hematoxylin and Eosin, and van Gieson's stain.

RESULTS

Histological changes of the urinary bladder epithelium were classified into 3 types, hyperplasia, papilloma, and cancer, the transitional cell carcinoma, as described previously.10,15) Cancerous lesions of the liver, including cholangiocarcinoma and hepatocellular carcinoma, were classified as cancer, but cholangiofibrosis and hyperplastic nodules were not. No remarkable changes were seen in the kidneys or other organs.

Experimental Series I  The average changes in body and liver weights and histological findings in the urinary bladder and in liver tumors in rats in each group are summarized in Table I. All the rats gained weight but the increase was not marked in groups pre-treated with N-nitrosomorpholine. The liver weight and its percentage to body weight increased slightly in the groups administered N-nitrosomorpholine, while other groups showed no remarkable changes in body and liver weights. Liver cancers developed in the groups treated with N-nitrosopiperidine, N-nitrosomorpholine, or diethylnitrosoamine but not in the groups treated with N-2-fluorenylacetamide or N-butyl-N-(4-hydroxybutyl)nitrosoamine alone.

Changes in the urinary bladder epithelium were seen in the rats treated with N-butyl-N-(4-hydroxybutyl)nitrosoamine or N-nitrosopiperidine. In rats treated with N-nitrosopiperidine alone, hyperplasia in the urinary bladder epithelium was seen in about 50~70% of the rats, but no papillomas or cancers were seen. On treatment with N-butyl-N-(4-hydroxybutyl)nitrosoamine, only 9 of 19 rats (47.4%) developed cancer in the urinary bladder epithelium. In the groups pre- or post-treated with chemical carcinogens as well as N-butyl-N-(4-hydroxybutyl)nitrosoamine, the percentages of urinary bladder cancer were lower. Except in the group treated with diethylnitrosoamine, the incidences of hyperplasias or papillomas in the urinary bladder epithelium on pre- or post-treatment with various carcinogenic chemicals were in general slightly lower than in the group treated with N-butyl-N-(4-hydroxybutyl)nitrosoamine alone. Histological patterns of hyperplasias, papillomas, or cancers of the urinary bladder and liver tissues were not influenced by treatment with a combination of N-butyl-N-(4-hydroxybutyl)nitrosoamine and other chemical carcinogens.

Experimental Series II  Changes in the body and liver weights and histological findings in the urinary bladder epithelium of the rats in each group are summarized in Table II. All the rats gained weight and no liver or kidney neoplasias were seen. Changes in the urinary bladder epithelium were noticed in rats treated with N-butyl-N-(4-hydroxybutyl)nitrosoamine and several chemicals. The induction of hyperplasia in the bladder epithelium by N-butyl-N-(4-hydroxybutyl)nitrosoamine was remarkably inhibited by pre-treatment with 1-naphthyl isothiocyanate for 8 weeks, but other chemicals tested had no inhibitory effect on the production of hyperplasia in the urinary bladder epithelium. The production of cancers in the urinary bladder epithelium was also clearly inhibited by 1-naphthyl isothiocyanate and 4-chloroacetanilide but not by other two chemicals. In contrast, pre-treatment with N-(3,5-dichlorophenyl)-
Table I. Effect of Pre- or Post-treatment with Various Carcinogens on Induction of Urinary Bladder Tumors in Rats by N-Butyl-N-(4-hydroxybutyl)nitrosoamine

<table>
<thead>
<tr>
<th>Treatment with carcinogen (weeks)</th>
<th>No. of rats</th>
<th>Body weight (g)</th>
<th>Changes in liver weight (% of body wt.)</th>
<th>Changes in urinary bladder&lt;sup&gt;b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial Final</td>
<td>Cancer</td>
<td>Hyperplasia Papilloma Cancer</td>
</tr>
<tr>
<td>Pre- (4) BBN (8) Post- (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>158.7 375.6</td>
<td>9.7 2.6 0</td>
<td>19 (100.0) 18 (94.7) 9 (47.4)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>158.8 428.9</td>
<td>10.4 2.4 0</td>
<td>10 (100.0) 7 (70.0) 3 (30.0)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>170.1 450.4</td>
<td>11.6 2.6 0</td>
<td>7 (50.0) 0 0 0</td>
</tr>
<tr>
<td>NNP</td>
<td>11</td>
<td>161.6 409.8</td>
<td>11.6 2.8 0</td>
<td>10 (90.9) 8 (72.7) 5 (45.5)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>183.4 457.1</td>
<td>17.2 2.7 1</td>
<td>5 (71.4) 0 0 0</td>
</tr>
<tr>
<td>NNP</td>
<td>11</td>
<td>168.3 404.0</td>
<td>10.3 2.5 0</td>
<td>11 (100.0) 7 (63.5) 1 (9.1)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>171.9 414.1</td>
<td>11.4 2.8 2</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>NNM</td>
<td>15</td>
<td>167.7 234.8</td>
<td>8.0 3.4 3</td>
<td>14 (93.3) 11 (73.3) 3 (20.0)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>180.1 280.7</td>
<td>7.4 2.7 1</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>172.8 322.4</td>
<td>8.0 2.5 0</td>
<td>14 (100.0) 14 (100.0) 6 (42.9)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>182.3 346.5</td>
<td>9.0 2.6 1</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>DEN</td>
<td>10</td>
<td>195.5 439.8</td>
<td>11.2 2.6 1</td>
<td>9 (90.0) 6 (60.0) 1 (10.0)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>204.0 520.1</td>
<td>13.8 2.7 3</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>176.7 393.8</td>
<td>10.5 2.7 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>2-FAA</td>
<td>11</td>
<td>194.7 445.4</td>
<td>11.7 2.6 0</td>
<td>10 (90.1) 10 (90.1) 3 (27.3)</td>
</tr>
<tr>
<td>2-FAA</td>
<td>10</td>
<td>168.1 354.7</td>
<td>9.3 2.6 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>157.3 460.1</td>
<td>12.4 2.7 0</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> BBN = 0.05% N-butyl-N-(4-hydroxybutyl)nitrosoamine in drinking water, NNP = 0.03% N-nitrosopiperidine in diet, NNM = 0.015% N-nitrosomorpholine in drinking water, DEN = 0.005% diethylnitrosamine in drinking water, 2-FAA = 0.025% N-2-fluorenylacetamide in diet; — = not treated with BBN, + = treated with BBN. Rats were killed 8 weeks after administration of carcinogens.

<sup>b</sup> Number of animals and percentages in parentheses.
succinimide and dimethylnitrosoamine slightly promoted the production of papilloma and cancer in the urinary bladder epithelium.

**Experimental Series III** Changes in the body and liver weights and histological findings in the liver and urinary bladder of the rats in each group are summarized in Table III. Groups treated with N-2-fluorenylacetamide developed liver cancer and hyperplastic nodules in the liver, but other groups did not.

No changes in the urinary bladder epithelium were observed in rats which received 0.001% N-butyl-N-(4-hydroxybutyl)nitrosoamine or 0.005% N-2-fluorenylacetamide for 40 weeks. However, 9 of the 11 rats (81.9%) which received both N-butyl-N- (4-hydroxybutyl)nitrosoamine and N-2-fluorenylacetamide for 40 weeks developed hyperplasias and, in the same group, 6 of the 11 rats (54.5%) developed papillomas and 1 (9.1%) developed cancer. Groups treated with the two carcinogens plus DL-tryptophan showed urinary bladder changes similar to rats treated with a combination of the two carcinogens only. However, induction
of hyperplasias of the urinary bladder epithelium by N-butyl-N-(4-hydroxybutyl)nitrosoamine or N-2-fluorenylacetaminde was slightly promoted by the administration of DL-tryptophan. No remarkable changes were seen in rats treated with DL-tryptophan alone for 40 weeks.

DISCUSSION

The present results show the effect of different chemicals on urinary bladder tumorigenesis in rats treated with N-butyl-N-(4-hydroxybutyl)nitrosoamine. In general, many chemicals used except N-butyl-N-(4-hydroxybutyl)nitrosoamine for the present study have hepatotoxic or nephrotoxic actions.\(^{12,18,20,23}\) Okada et al.\(^{6,9,25}\) presumed that N-butyl-N-(4-hydroxybutyl)-nitrosoamine may be metabolized to some of proximate carcinogens in the rat liver, and it was suggested that the production of urinary bladder cancer in rats by N-butyl-N-(4-hydroxybutyl)-nitrosoamine should be changed by pre- or post-treatment with hepatotoxic or nephrotoxic chemicals.

In experimental series I, the influence of different chemical carcinogens given before or after the administration of N-butyl-N-(4-hydroxybutyl)nitrosoamine was investigated. These carcinogenic chemicals had little effect on urinary bladder tumorigenesis induced by N-butyl-N-(4-hydroxybutyl)nitrosoamine, and did not inhibit production of papillomas and cancers in urinary bladder induced by the latter.

In experimental series II, the effect of other chemicals was investigated. 4-Chloroacetanilide and N-(3,5-dichlorophenyl)succinimide are both nephrotoxic but not carcinogenic.\(^{18,32,33}\) 1-Naphthyl isothiocyanate has a hepatotoxic action in rats and, on chronic administration, induced liver bile duct proliferation or biliary cirrhosis.\(^{12,13,20,22,31,37}\) Diethylnitrosamine has hepatotoxic and hepatocarcinogenic actions.\(^{23,24,36}\) The present results showed that development of urinary bladder cancer in rats by N-butyl-N-(4-hydroxybutyl)nitrosoamine was inhibited by 4-chloroacetanilide or 1-naphthyl isothiocyanate. Previously, it was suggested that focal hyperplasia of the bladder epithelium is a precancerous lesion.\(^{14,15}\) In the present work, development of hyperplasia of the urinary bladder epithelium was only inhibited in the group receiving 1-naphthyl isothiocyanate and no remarkable differences were seen in other groups, including those that received 4-chloroacetanilide. These results show that only 1-naphthyl isothiocyanate had any marked inhibitory effect on urinary bladder tumorigenesis in rats induced by N-butyl-N-(4-hydroxybutyl)nitrosoamine. Previously, it was reported that induction of liver cancer in rats by 3'-methyl-4-((dimethylamino)azobenzene, N-2-fluorenylacetamide, DL-ethionine, m-toluylenediamine, or diethylnitrosamine was clearly inhibited by 1-naphthyl isothiocyanate.\(^{12,31,24}\) The inhibitory effect of 1-naphthyl isothiocyanate on chemical carcinogens was observed not only in the liver but also in the urinary bladder. The mechanism of these inhibitory actions are still unknown but, it is presumed that the effect of liver changes, as a result of hepatotoxic action, should influence urinary bladder tumorigenesis of N-butyl-N-(4-hydroxybutyl)nitrosoamine in rats.

In experimental series III, the interaction of two different carcinogens in urinary bladder tumorigenesis in rats was analysed. Non-carcinogenic doses of both N-butyl-N-(4-hydroxybutyl)nitrosoamine and N-2-fluorenylacetamide were used.\(^{3,5,6,17,29,30}\) These results showed that a combination of N-butyl-N-(4-hydroxybutyl)nitrosoamine and N-2-fluorenylacetamide promoted urinary bladder tumorigenesis in rats. In contrast, N-2-fluorenylacetamide did not promote induction of urinary bladder cancer in rats by N-butyl-N-(4-hydroxybutyl)-nitrosoamine in experimental series I. These findings showed that the result depends on whether these two carcinogens are administered at the same time or at different times. Nakahara et al. observed a summation of the effects of different carcinogen sin several organs.\(^{25}\) Many
BLADDER TUMORS BY BBN

observers have also reported the synergistic actions of hepatocarcinogenesis in rats.12,21,24) Our results show that N-butyl-N-(4-hydroxybutyl)nitrosoamine and N-2-fluorenylacetamide have synergistic action in urinary bladder tumorigenesis in rats. Previously, Okajima et al.27,28) reported the synergistic effect of DL-tryptophan and N-butyl-N-(4-hydroxybutyl)nitrosoamine or N-dibutylnitrosoamine, and Dunning et al.5,6) showed that tryptophan increased the incidence of urinary bladder tumors in rats induced by N-2-fluorenylacetamide. Our results confirm these previous findings on the carcinogenic effect of N-butyl-N-(4-hydroxybutyl)-nitrosoamine and N-2-fluorenylacetamide on the urinary bladder of rats.

The present work shows that analysis of the synergistic or antagonistic effect of chemicals, with both known and unknown carcinogenic actions, may be important in the protection of bladder cancer in man.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education (No. 90319/1972 and No. 801062/1973), by a grant from the Tokyo Biochemical Research Foundation, and by a grant from the Experimental Pathological Research Association (1973). These grants are gratefully acknowledged.

(Received October 1, 1973)

REFERENCES

18) Ito, N., Sugiara, S., unpublished data.
36) Takayama, S., Oota, K., Gann, 56, 189 (1965).