Urethan Carcinogenesis and Nucleic Acid Metabolism: Factors Influencing Lung Adenoma Induction

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Summary

Experiments were performed (a) to test the validity of the proposal that urethan (ethyl carbamate) acts by virtue of its conversion to N-hydroxyurethan, and (b) to examine the generality of the reported observations that pyrimidines interfere with lung adenoma formation by urethan.

Conditions were obtained in adult and newborn SWR mice in which the average number of tumors per mouse increased linearly with urethan dose; the number of spontaneous tumors appearing in untreated mice was negligible compared with the number induced by urethan. A comparison of urethan and N-hydroxyurethan, as inducers of lung adenomas when injected into newborn mice, showed that, at low dose levels, N-hydroxyurethan is several times less effective than urethan as a carcinogen. Furthermore, the drug potentiator SKF-525A, which inhibits the activity of certain oxidation-reduction enzymes in liver microsomes, had no effect on the induction of lung adenomas by urethan, but significantly inhibited the carcinogenic action of N-hydroxyurethan.

Thymidine, thymine, and orotic acid were all found to be ineffective in reducing the number of lung adenomas per mouse induced by a single injection of urethan, while aminopterin did not show any enhancing effect on lung adenoma formation by urethan. However, the reported reduction in spontaneous adenomas by urethan, when these were fed thymine in their drinking water, was confirmed.

It was concluded that urethan rather than N-hydroxyurethan is the more likely proximal carcinogen and that no consistent or general direct effect of pyrimidines on urethan carcinogenesis has yet been established.

Introduction

Two related ideas are prevalent in speculation on the mechanism of urethan carcinogenesis: one idea is that there is a direct interaction between urethan and nucleic acids, and it is based mainly on biological antagonism experiments (6, 7, 13, 14, 30, 32); the other is that urethan itself is not carcinogenic but is converted in vivo into N-hydroxyurethan (8, 22) a compound previously shown to be carcinogenic (2, 22). These 2 ideas find mutual support in that N-hydroxyurethan is a more reactive compound than urethan and is more likely to interact with nucleic acids (5). When N-hydroxyurethan was first shown to be carcinogenic by Berenblum et al. (2), but to have less than half the potency of urethan, it was considered unlikely that urethan acted by conversion to N-hydroxyurethan. However, the finding that metabolic N-hydroxylation increased the carcinogenicity of acetylaminofluorene led Miller et al. (22) to propose that N-hydroxyurethan is indeed the proximal carcinogen. Boyland and Nery have supported this viewpoint and recently reported that they have detected N-hydroxyurethan in the urine of rats, rabbits, and man after injection of urethan (8).

Speculations linking the biologic activity of urethan to the inhibition of a step in nucleic acid synthesis date from 1946, when Haddow and Sexton (16) quoted a suggestion of A. R. Todd that urethan’s growth-inhibiting action might be due to the induction of a deficiency in purine synthesis. Dustin (12) suggested that urethan’s antimitotic action might be due to “inactivation of enzymes playing a part in the onset of prophase” (in particular, thymonucleoprotein synthesis). Subsequently, mutagenic activity of urethan was reported (see Ref. 35 for recent summary).

The 1st biochemical indication that urethan inhibited nucleic acid synthesis arose from a study of possible inhibitors of tumor growth by Skipper et al. (34). Rogers (30) reported that sodium deoxyribonucleate inhibited lung adenoma formation by urethan, and Boyland and Koller (7) found that thymine inhibited the chromosone-damaging action of urethan. From biologic antagonism experiments, Rogers (32) reported, among other effects, that the induction of lung adenomas in mice by urethan was potentiated by aminopterin, adenine, and oxaloacetic acid, inhibited by thymine, asparagine, and orotic dihydroorotic, ureidosuccinic and cytidylic acids, and not influenced by thymidylic acid. He suggested that urethan acts “in the pathway of nucleic acid synthesis below orotic acid, and perhaps at the level of ureidosuccinic acid.” Fink and Fink (15) reported a decrease in spontaneous lung tumor incidence in mice when given 0.4% thymine in their drinking water. An antagonism to the tumor-inhibiting action of urethan by thymidine and thymine was found by Elion and her associates (13, 14), in a chemotherapeutic study using mammary adenocarcinoma 755. They also reported an inhibition by urethan of the incorporation of uracil-2-14C into mouse liver, gut, and adenocarcinoma. Handschumacher and Welch (17) have critically discussed the evidence linking urethan to nucleic acid metabolism and concluded: “much more work will be needed to explain satisfactorily the remarkable effects of this compound on nucleic acid metabolism.”

In an attempt to extend the observations of Rogers (32), Nechama Haran-Ghera of this department tested orotic acid and thymine, among other nucleic acid precursors and metabolic intermediates, for their ability to interfere with urethan’s potency.

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2 Herbert Sidebotham Research Associate.

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in initiating skin papillomas or causing lung adenomas, and found that none of these compounds significantly altered the response of Swiss mice to urethan (unpublished results).

In order to clarify the relationship of urethan to nucleic acid metabolism, the experiments reported in this paper were designed to answer the questions whether (a) N-hydroxyurethan rather than urethan is the proximal carcinogen; (b) there is an antagonism between urethan and pyrimidines in lung adenoma induction; and whether (c) thymine suppresses spontaneous lung adenoma formation in strain A mice.

Materials and Methods

Urethan (ethyl carbamate) was obtained from British Drug Houses, Ltd.; thymine, thymidine, and orotic acid from Calbiochem, Inc.; and aminopterin from Eli Lilly & Co. Thymine was also obtained from Schwarz Biochemicals, Inc., and for comparative purposes, samples of thymine and orotic acid from Nutritional Biochemicals Corp. were also tested. N-hydroxyurethan was synthesized and purified (by redistillation) by S. Mirvish of this laboratory. SKF-525A was a gift of Smith, Kline and French Labs.

SWR/J mice were bred at the Weizmann Institute of Science by sibling mating. A/He mice were obtained from the Jackson Memorial Laboratory. Young adult mice were vaccinated against ectromelia. Newborn SWR mice separated from their mothers, as well as young adult mice, were randomized among the experimental groups and kept in an air-conditioned room at 21-24°C. Their diet consisted of Purina laboratory chow pellets, supplemented by barley and sunflower seeds, and water ad libitum.

Chemicals were dissolved or suspended in distilled water and injected on the basis of the body weight of individual mice. Newborn mice (1.1-1.7 gm) were given s.c. injections of urethan or N-hydroxyurethan, using a No. 27 hypodermic needle, at a concentration at which they received 0.05 ml of solution per gm. In adult mice, urethan was injected i.p. either as a 5 or 10% or as a 5 mM solution, N-hydroxyurethan as a 5 mM solution, SKF-525A as a solution of 5 mg/ml, thymine as a 2% suspension, thymidine as a 4% solution, and aminopterin as a solution of 25 µg/ml. Animals were given thymidine to drink as a 0.8% solution and thymine as a 0.4% solution, both in tap water. To serve as controls for the i.p. injected adult mice, animals were given injections of the same volume of 0.9% NaCl; controls for the mice which received thymidine or thymine in their drinking water were untreated. Newborn mice were kept separated from their mothers for several hours after injection, after which period all anesthetic effects had worn off. Mothers were then chosen at random for each of the groups. The survival rate of newborn mice with foster mothers was no lower than that of mice with their own mothers.

All adult mice were weighed weekly to detect any consistent significant difference in the average weights of the experimental and control groups. Only in groups of mice receiving aminopterin were such differences found. At a predetermined time after the beginning of the experiment the mice were autopsied and examined under a dissecting microscope for the presence of lung adenomas. Histologic sections of all questionable tumors were examined for confirmatory diagnosis. Using a table of "t" values, 95% confidence intervals were calculated, and the significance of differences in incidence of tumors was evaluated by means of the χ² test.

Results

Characteristics of the Test System

Results of experiments aimed at defining the test system and verifying that the doses of urethan (usually 1 mg/gm or 0.5 mg/gm) which had been employed by Rogers (32) were optimal for revealing possible inhibitory effects in the present experimental system are summarized in Charts 1–3. In young adult SWR mice, a dose of 1 mg of urethan/gm of mouse resulted 12 weeks later in adenomas in all 57 mice autopsied. Half this dose (0.5 mg/gm) produced 76% incidence (Chart 1). The number of adenomas per mouse increased 60-fold, linearly with dose, in the range 0.25–1.0 mg of urethan/gm (Chart 2) after an apparent threshold dose was exceeded. Seven weeks after urethan injection, the earliest
time investigated, macroscopic adenomas were visible. In response to a dose of 0.5 mg/gm of urethan, multiple tumors appeared within 10 weeks after urethan injection, when the mice were less than 20 weeks old. The number of spontaneous adenomas per mouse reached a significant level only at the end of the 1st year of life (Chart 3). In male control mice, only single tumors appeared in animals killed within 1 year of birth, and only in animals killed at 80 weeks were 2 adenomas found in the same mouse.

Injection or 2 μmoles/gm (0.18 mg/gm) of urethan into newborn mice produced, 10 weeks later, adenomas in all mice autopsied (Chart 1) with a yield of more than 2 adenomas/mouse per mg/gm increment in urethan dose, whereas a slightly higher dose (0.25 mg/gm) given to young adult mice produced no tumors (Chart 1). The same pattern of an apparent threshold followed by a linear response was shown by the newborn and adult mice (Chart 2). If the difference in response is expressed as a rate, from linear portions of the curves in Chart 2 it can be calculated that while young adult mice show 4.9 adenomas/mouse per mg/gm increment in urethan dose, within 12 weeks, the newborn mice respond with 18.2 adenomas/mouse for the same urethan increment, within 10 weeks.

Comparison of Carcinogenicity of Urethan and N-Hydroxyurethan

In female weanling mice (9–10 weeks old) doses of 5 μmoles/gm (0.45 mg/gm) and 10 μmoles/gm of urethan and N-hydroxyurethan show N-hydroxyurethan to be 3 to 4 as potent as urethan (Table 1, Groups 1–4). However, on reducing the dose and using newborn mice (in which urethan is more effective than in older mice) the relative effectiveness of N-hydroxyurethan was seen to be much lower when it was administered in doses equimolar with those of urethan, as reported in Charts 1 and 2 for newborn mice. At a dose of 2 μmoles/gm (0.18 mg of urethan), urethan induced more than 5 times as many lung adenomas in 10 weeks (average = 2.3, 95% confidence interval = 1.8–2.7) as did N-hydroxyurethan (average = 0.4, 95% confidence interval = 0.0–0.9).

In the presence of the drug SKF-525A (β-dimethylaminooethyl-diphenylpropylacetate), which inhibits the conversion of N-hydroxyurethan to urethan (24), no effect was found on urethan’s ability to induce lung adenomas (Table 1, groups 3 and 5), but N-hydroxyurethan’s potency was reduced by a factor of 3 (Table 1, Groups 4 and 6).

Attempts to Modify the Carcinogenicity of Urethan

Thymine and thymidine showed no inhibition of urethan-induced adenoma formation (Table 2) when given simultaneously with urethan injections (cf. Long-term Thymine Administration, below). Administration in the animals’ drinking water of several times the dose of thymidine injected (Table 2, Group 5) led to the same adenoma yield. Since Groups 5 and 6 were starved for 45 hr, a comparison can be made between the adenoma yield following urethan administration to fully fed (Groups 1–4) and starved mice. No effect of starvation on lung adenoma production was demonstrated. The age of the mice did, however, influence the tumor yield, which showed a consistently higher trend in younger mice, even within the young adult range.

Urotic acid, administered to both fully fed and starved mice of different ages (killed at 10 or 12 weeks after urethan injection) showed no inhibitory effect on urethan’s tumorigenic potency. In fact, a slight, but insignificant, increase in tumor yield can be noted in all 4 experiments reported in Table 3. Aminopterin administration (Groups 9, 11, Table 3) caused no difference in the yield of lung adenomas observed 10 weeks after urethan injection. The average weights of the aminopterin-treated groups were 0.5 and 1.0 gm lower than their control groups.

Spontaneous Lung Adenomas in A/HeJ Mice

Preliminary experiments showed that the spontaneous lung adenoma yield in A/HeJ male mice increased from 0.1 adenoma/mouse in 9-month-old mice to 0.6 adenoma/mouse in 10-mouth-old mice.

<table>
<thead>
<tr>
<th>CARCINOGEN (μmoles/gm)</th>
<th>TEST AGENT</th>
<th>NO. OF MICE</th>
<th>AV. AGE AT INJECTION (wk.)</th>
<th>SURVIVORS</th>
<th>ADENOMA INCIDENCE (%)</th>
<th>ADENOMAS/SURVIVOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Urethan, 5</td>
<td>None</td>
<td>25</td>
<td>9</td>
<td>23</td>
<td>57</td>
<td>1.0</td>
</tr>
<tr>
<td>2. N-hydroxyurethan, 5</td>
<td>None</td>
<td>25</td>
<td>9</td>
<td>22</td>
<td>27</td>
<td>0.4</td>
</tr>
<tr>
<td>3. Urethan, 10</td>
<td>None</td>
<td>25</td>
<td>10</td>
<td>25</td>
<td>100</td>
<td>4.0</td>
</tr>
<tr>
<td>4. N-hydroxyurethan, 10</td>
<td>None</td>
<td>22</td>
<td>10</td>
<td>20</td>
<td>75</td>
<td>1.9</td>
</tr>
<tr>
<td>5. Urethan, 10</td>
<td>SKF-525A</td>
<td>24</td>
<td>10</td>
<td>23</td>
<td>96</td>
<td>4.1</td>
</tr>
<tr>
<td>6. N-hydroxyurethan, 10</td>
<td>SKF-525A</td>
<td>22</td>
<td>10</td>
<td>21</td>
<td>62</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Solutions were injected i.p. and mice were killed 10 weeks later. SKF-525A was administered at a dose rate of 50 μg/gm.

CHART 3. Spontaneous lung adenomas found in SWR male mice at different ages. Vertical lines indicate 95% confidence intervals. (N.B. The abscissa in this chart is drawn to the same scale as in Chart 2.)
old males. In females, the yield was also 0.1 adenoma/mouse at 9 months, but only doubled by 10 months, reaching 1.1 adenoma/female at 12 months. Therefore, in this experiment on tumor inhibition, males were killed at the age of 10 months and females at 1 year. Mice, from the age of 7-9 weeks till they were killed, were given 0.4% thymine (Schwarz BioResearch, Inc.) in their drinking water, which resulted in a daily intake of approximately 20 mg of thymine/mouse. In 21 normal mice, a tumor incidence of 48% (0.81 adenomas/mouse) was found, while in 48 thymine-fed mice the incidence was reduced to 17% (0.21 tumors/mouse).

### TABLE 2

**Effect of Thymine and Thymidine on Lung Adenoma Induction by Urethan in SWR Mice**

<table>
<thead>
<tr>
<th>URETHAN (1 mg/gm)</th>
<th>TEST AGENT</th>
<th>NO. OF MICE</th>
<th>SEX</th>
<th>AV. AGE AT INJECTION (wk.)</th>
<th>SURVIVORS</th>
<th>ADENOMAS/SURVIVORS Mean</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. i.p.</td>
<td>Thymine, 0.4 mg/gm i.p.</td>
<td>16</td>
<td>♂</td>
<td>22</td>
<td>15</td>
<td>4.0</td>
<td>2.6-5.5</td>
</tr>
<tr>
<td>2. i.p.</td>
<td>Control</td>
<td>20</td>
<td>♂</td>
<td>22</td>
<td>20</td>
<td>3.5</td>
<td>2.9-4.0</td>
</tr>
<tr>
<td>3. i.p.</td>
<td>Thymidine, 0.8 mg/gm i.p.</td>
<td>20</td>
<td>♂♀</td>
<td>10</td>
<td>19</td>
<td>6.2</td>
<td>4.7-7.6</td>
</tr>
<tr>
<td>4. i.p.</td>
<td>Control</td>
<td>19</td>
<td>⿰♀</td>
<td>10</td>
<td>18</td>
<td>4.3</td>
<td>3.1-5.4</td>
</tr>
<tr>
<td>5. i.p. to fasted mice</td>
<td>Thymidine, 0.8% p.o.</td>
<td>10</td>
<td>♂</td>
<td>12</td>
<td>9</td>
<td>3.6</td>
<td>2.1-5.1</td>
</tr>
<tr>
<td>6. i.p. to fasted mice</td>
<td>Control</td>
<td>10</td>
<td>♂</td>
<td>12</td>
<td>9</td>
<td>4.0</td>
<td>3.5-4.5</td>
</tr>
</tbody>
</table>

*Urethan was injected as a 10% solution in distilled water at the same time as the test agent. Thymine was given as a 2% suspension in distilled water in Group 1. Thymidine was given as a 4% solution in Group 3 and as a 0.8% solution in tap water (serving as drinking water) in Group 5. In this group the mice were fasted for 24 hr before injection of urethan and 21 hr after injection. During this 45-hr starvation period the thymidine was administered. The control solution injected in Groups 2 and 4 was 0.9% NaCl. In Group 6, mice were given tap water to drink. The animals were killed 12 weeks after urethan injection.*

### TABLE 3

**Effect of Orotic Acid or Aminopterin on Lung Adenoma Induction by Urethan in Male SWR Mice**

<table>
<thead>
<tr>
<th>URETHAN (mg/gm)</th>
<th>TEST AGENT</th>
<th>NO. OF MICE</th>
<th>AV. AGE AT INJECTION (wk.)</th>
<th>DURATION OF EXPERIMENT (wk.)</th>
<th>SURVIVORS</th>
<th>ADENOMA INCIDENCE (%) Mean</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1</td>
<td>Orotic acid, 0.1 mg/gm</td>
<td>10</td>
<td>14</td>
<td>12</td>
<td>5</td>
<td>100</td>
<td>4.6</td>
</tr>
<tr>
<td>2. 1</td>
<td>Control</td>
<td>10</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>100</td>
<td>3.8</td>
</tr>
<tr>
<td>3. 0.5</td>
<td>Orotic acid, 0.2 mg/gm</td>
<td>12</td>
<td>13</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>3.5</td>
</tr>
<tr>
<td>4. 0.5</td>
<td>Control</td>
<td>12</td>
<td>13</td>
<td>10</td>
<td>12</td>
<td>83</td>
<td>1.8</td>
</tr>
<tr>
<td>5. 0.5</td>
<td>Orotic acid, 0.2 mg/gm</td>
<td>8</td>
<td>22</td>
<td>12</td>
<td>7</td>
<td>71</td>
<td>1.3</td>
</tr>
<tr>
<td>6. 0.5</td>
<td>Control</td>
<td>9</td>
<td>22</td>
<td>12</td>
<td>7</td>
<td>71</td>
<td>1.4</td>
</tr>
<tr>
<td>7. 0.5</td>
<td>Orotic acid, 0.2 mg/gm</td>
<td>23</td>
<td>10</td>
<td>12</td>
<td>19</td>
<td>75</td>
<td>1.3</td>
</tr>
<tr>
<td>8. 0.5</td>
<td>Control</td>
<td>22</td>
<td>10</td>
<td>12</td>
<td>22</td>
<td>76</td>
<td>1.1</td>
</tr>
<tr>
<td>9. 0.5</td>
<td>Aminopterin, 1.25 µg/gm</td>
<td>11</td>
<td>15</td>
<td>10</td>
<td>10</td>
<td>90</td>
<td>1.6</td>
</tr>
<tr>
<td>10. 0.5</td>
<td>Control</td>
<td>12</td>
<td>15</td>
<td>10</td>
<td>11</td>
<td>82</td>
<td>1.7</td>
</tr>
<tr>
<td>11. 0.5</td>
<td>Aminopterin, 1.25 µg/gm</td>
<td>24</td>
<td>13</td>
<td>10</td>
<td>19</td>
<td>74</td>
<td>1.6</td>
</tr>
<tr>
<td>12. 0.5</td>
<td>Control</td>
<td>23</td>
<td>13</td>
<td>10</td>
<td>19</td>
<td>90</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Urethan was injected i.p. at the same time as the last injection of the test agent. Orotic acid was given as a 1% suspension in distilled water. In Group 1, a single injection was given; in Groups 3, 5, and 7, 2 injections of 0.1 mg/gm were given with a 24-hr fast intervening. Aminopterin was injected as a solution of 25 µg/gm. Three daily injections of 0.25 µg/gm were followed by 1 injection of 0.5 µg/gm. Control injections consisted of 0.9% NaCl equal in volume to the test solutions.*
The number of tumor-bearing mice in the 2 groups is significantly different ($P = 0.02$), and the difference in the yield of tumors/mouse is highly significant ($P < 0.001$).

**Long-term Thymine Administration to Urethan-injected SWR Mice**

In the light of the inhibition of spontaneous lung adenoma incidence by thymine in A/He mice, a similar experiment on urethan-induced tumors was performed. A batch of 5-week-old SWR male mice was injected with 0.5 mg of urethan/gm of body weight. Group 1 (control) received no other treatment. Group 2 was given 0.4% thymine in its drinking water 1 day prior to and 2 days following urethan injection. Four days after urethan injection, the mice of Group 3 were given drinking water containing 0.4% thymine, this treatment continuing for 10 weeks until the animals were killed. The tumor yields (0.71, 1.0, and 1.18 adenomas/mouse in Groups 1, 2, and 3, respectively) showed no inhibition of the tumorigenic effect of urethan by thymine.

**Discussion**

The question of whether urethan or N-hydroxyurethan is the active carcinogen is crucial for the interpretation of in vivo experiments on urethan-induced tumor formation. The biochemical studies in this laboratory by Mirvish (23), which showed that in SWR mice 70% of an injected dose of 1 mg/gm of N-hydroxyurethan-$^{14}$C is converted to urethan, while the reverse reaction is not detectable in these animals, confirmed earlier studies from this laboratory (3, 18), which showed no evidence for a carcinogenic urethan metabolite in mice, and led to the viewpoint that N-hydroxyurethan is active as a carcinogen by virtue of its N-dehydroxylation to urethan (4, 24). Since N-hydroxyurethan disappears more rapidly than urethan from the blood of mice (23), it was predicted, and confirmed in the present work, that the relative conversion of N-hydroxyurethan to urethan would become lower as the dose of urethan was decreased and that the apparent carcinogenicity of N-hydroxyurethan relative to urethan would also decrease. Thus, high dosages (repeated injections) of urethan and N-hydroxyurethan showed no difference in their carcinogenic potency (4); medium dosages (a single injection of the most widely used concentrations) of these agents showed urethan to be 2-3 times as effective as N-hydroxyurethan (Table 1; Refs. 1, 19), while, in the present study, low doses of these agents in newborn mice showed that urethan induced more than 5 times the number of lung adenomas than an equimolar dose of N-hydroxyurethan.

However, a more direct test for the proximal carcinogen was made possible by the use of SKF-525A, a drug which interferes with certain oxidation-reduction reactions taking place in the microsomal fraction of liver (1, 9), and which has recently been found, by Mirvish (24), to inhibit the dehydroxylation of N-hydroxyurethan to urethan. The inhibition of N-hydroxyurethan carcinogenesis by SKF-525A, found in the present work, coupled with the lack of effect of this drug on urethan itself and with the considerations mentioned above lead to the conclusion that urethan rather than N-hydroxyurethan is the more proximal carcinogen.

Having reached this conclusion, the pertinent question is the capability of urethan itself to interact with nucleic acid metabolism. To clarify this problem, the following compounds were tested by Haran-Ghera for their ability to interfere with urethan carcinogenesis (both skin papilloma and lung adenoma formation) in Swiss mice, using the techniques employed in this department (2); DNA, RNA; adenine, guanine, diaminopurine, xanthine, hypoxanthine, thymine, cytidine, uracil; orotic, uridine nucleosine, L-aspartic, uric, L-malic, ketoglutaric, and glutamic acids; the sodium salts of formic, oxaloacetic, fumaric, and succinic acids; and methionine, ethionine, and pyridoxal chloride. All were shown to be without significant effect on urethan carcinogenesis (Haran-Ghera, unpublished results). These observations on Swiss mice, inconsistent with the results of Rogers (32), who used mice of Swiss, A, and C strains, led to the present, and to a parallel, series of biochemical experiments using SWR mice, neither of which furnished evidence for an interaction between urethan and nucleic acid metabolism which might explain urethan's carcinogenicity. Some of the experiments on in vitro enzyme systems and on incorporation in vivo of $^{14}$C-labeled nucleic acid precursors have been reported briefly (20) and will be described in subsequent papers.

The adequacy of choice of a test system (Charts 1—3) for investigating possible inhibition of urethan-induced adenoma formation was shown by the reduction of the control variation in average tumors/mouse given 1 mg of urethan/gm from experiment to experiment (from more than 10-fold for Swiss and C mice and more than 4-fold for A mice as reported by Rogers (32) to a value of 1.3-fold).

Thymine and orotic acid were chosen for this study since they were the most effective compounds reported by Rogers to interfere with urethan-induced adenoma formation (32). Orotic acid was also reported to reduce the tumor incidence following administration of methylcholanthrene (31). In the experiments reported here, thymine was administered by i.p. injection, in drinking water during a 45-hr fast, or either for 4 days during the possible “initiating” period of urethan carcinogenesis (a much longer time than urethan remains in the body of young adult mice (19, 25)), or for the period from 4 days after urethan injection to autopsy of the animals. Under none of these conditions was the induction of adenomas by urethan diminished (see Table 2 and section on Long-term Thymine Administration). Because thymidine is more efficiently utilized than thymine as a precursor of DNA (10) and since Boyland (6) reported that it was more effective than thymine in preventing the chromosome-damaging action of urethan, it was also tested and found unable to reduce the induction of adenomas by urethan (Table 1). Previous work (35) had shown no effect of thymidine (or adenine) on formation of skin papillomas in Swiss mice subsequently painted with croton oil.

Orotic acid was tested in 2 concentrations, and at 2 urethan levels, in mice of varying ages in experiments of 10 or 12 weeks' duration (Table 2). No effect on lung adenoma induction was found, nor was the reported (32) potentiation of lung adenoma formation by aminopterin confirmed in 2 independent experiments (Table 3). However, the observation of Fink and Fink (15) on the reduction of spontaneous adenomas in Strain A mice by thymine was completely confirmed.

As regards other factors reported to influence urethan carcinogenesis, our experiments showed no effect of starvation, despite conflicting reports on this point (28, 29). Our results did show an effect, first described by Rogers (28), confirming the difference in adenoma induction in mice given urethan at different ages.
Urethan Carcinogenesis

Evidence correlating this effect with the slower rate of urethan catabolism in younger mice has been presented previously (11, 19, 25). A significant increase in lung adenoma yield following urethan administration in thymectomized SWR mice has been observed very recently in this laboratory (Trainin, to be published).

The use of tumor formation by urethan, as a test system, giving an end result so distant in time from urethan's initial interaction in the body, increases the difficulty of reaching conclusions on biochemical mechanisms from biologic "reversal experiments" in living mice. The pitfalls in interpreting such reversal experiments, which were performed using the technic of skin painting, have been noted by Rogers (32). The confusing picture which led us to undertake the present investigation is made even more difficult to interpret in the light of additional complications (17), for example, the antagonism by dinamino purine of urethan's inhibition of Escherichia coli growth reported by Skipper et al. (34). In mice, many factors are critical in determining the final tumor yield. Rogers (personal communication) has recently made the plausible suggestion that nutritional factors may explain the different results on urethan induction of lung adenomas which have been obtained in his laboratory (28-30, 32) and in the present study. He pointed out that the Purina laboratory chow used in the present experiments contains 3.5 times as much folic acid as the Purina fox chow which he used. Liver meal and dried skimmed milk are also present in Purina laboratory chow but absent from the fox chow. In addition, animals in this laboratory receive supplementary feeds of barley and sunflower seeds. A significant difference in folic acid availability could thus explain why mice in the present study show less susceptibility to the same dose of aminopterin as the animals used by Rogers (32) and why supplementary pyrimidines may affect urethan carcinogenesis only in animals capable of nutritional improvement by such compounds.

Clarification of the apparent antagonisms between urethan and pyrimidines, reported in the literature (6, 7, 13, 14, 30, 32), is necessary in order to understand how this carcinogen interacts with a cellular component (which does not necessarily need to be a nucleic acid). The recent greatly increased understanding of the mechanism of enzyme induction and repression in bacteria and in higher organisms (26) has provided new ideas which give a crucial role to a repressor molecule, which is most likely a nucleoprotein, in the control of gene action. Pitot and Heidelberg (27) have shown that models for carcinogenesis can be made which result in tumor production without a direct change in DNA. In extension of our previous observations on the inhibition of proteolytic enzymes by urethan (21), Dr. A. Rimon from this Institute has found that thrombin acting on tosyl arginine methyl ester is inhibited by urethan (personal communication). If an irreversible binding of urethan to thrombin or to the serine of the active site of chymotrypsin is found, this linkage could be used as a model to search for specific interactions of urethan with cellular proteins which may be involved in carcinogenesis.

Since the results of the present investigation show no effect of pyrimidines on urethan carcinogenesis, one is led to the conclusion that no consistent specific or direct effect of urethan on nucleic acid synthesis has yet been established from in vivo experiments which can explain urethan's mechanism of action as a carcinogen.

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