Enhancement of Hepatocarcinogenesis in Female Rats by Ethinyl Estradiol and Mestranol but not Estradiol

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ABSTRACT

The effect of dietary exposure to synthetic estrogens on hepatocarcinogenesis was evaluated. Diethylstilbestrol-initiated and 0.85% NaCl solution-treated noninitiated female Sprague-Dawley rats were transferred to semisynthetic diets containing mestranol (0, 0.1, or 0.5 ppm), ethinyl estradiol (0.5 ppm), estradiol (0.6 ppm), or mestranol plus β-methasone (0.5 and 0.2 ppm, respectively). γ-Glutamyl transferase (GGT)-positive transsections and hematoxylin and eosin-detectable nodules and carcinomas were scored at 9 and 12 months. Quantitative stereological calculations were performed to determine GGT lesion number and size. At 9 months, in diethylstilbestrol-initiated rats, ethinyl estradiol and mestranol caused 3.5- and 4.4-fold increases, respectively, in the number of GGT lesions per liver and an increased incidence of hepatocellular carcinomas while estradiol had no enhancing effect. Addition of β-methasone to the mestranol-containing diet caused a significant decrease in GGT lesion number but not carcinoma incidence compared to mestranol alone. At 12 months, in diethylstilbestrol-initiated rats, mestranol caused a dose-dependent increase in GGT lesion number. The hepatocellular carcinoma incidence was significantly increased at the high mestranol dose. Small increases in the numbers of larger GGT lesions were also observed in noninitiated animals treated with mestranol and ethinyl estradiol and are most probably due to promotion of spontaneously initiated hepatocytes. These results indicate that the synthetic estrogens cause dramatic increases in the number of presumptive preneoplastic GGT lesions. Carcinoma incidence is also enhanced. Thus, these results confirm and extend our previous studies which together with the results of others have shown that synthetic estrogens can act as promoters of hepatocarcinogenesis.

INTRODUCTION

In humans, the evidence that use of oral contraceptive steroids is associated with an increased incidence of benign liver neoplasms is strong (see Refs. 36, 39, 40, and 43). While the results of several studies have also suggested an increased incidence of HC in women following prolonged use of these agents (11, 15, 19), the strength of this association remains controversial (11, 14) and deserves continued study.

In experimental studies in animals, results have been obtained which suggest that synthetic estrogen treatment following carcinogen exposure (initiation) can enhance hepatic neoplasia. Taper (32) was the first investigator to demonstrate that synthetic steroids used in oral contraceptive preparations in Europe, estradiol 17-phenylpropionate and estradiol benzoate, enhanced hepatocarcinogenesis in castrated female rats previously initiated with N-nitrosomorpholine. In the United States, 2 synthetic estrogens, mestranol (17β-ethinyl estradiol 3-methyl ether) and EE, are widely used in oral contraceptive preparations. We reported that, in intact, DEN-initiated female Sprague-Dawley rats, mestranol alone and together with norethynodrel enhanced the appearance of GGT-positive, presumptive preneoplastic foci (39, 40, 43). The data of Vesselinovitch and Mihailovich (33) demonstrated that mestranol enhanced the incidence and multiplicity of benign liver tumors in DEN-initiated female C57BL/6 x C3H F1 mice. Cameron et al. (3, 4) reported that EE enhanced the appearance of GGT foci and hyperplastic nodules in DEN-initiated male Fischer 344 rats. Wanless and Medline (34) also reported that EE enhanced the appearance of grossly visible hyperplastic nodules and microscopic hyperplastic foci in male Fischer 344 rats. They indicated that some of the grossly visible nodules were well-differentiated HCs but did not report the incidence of these neoplasms.

In 5 (3, 4, 32-34) of the studies mentioned above, initiation preceded treatment with the synthetic estrogens by a period ranging from 1 to 4 weeks. This suggests that the enhancement of hepatocarcinogenesis caused by the synthetic estrogens is due to promotion (Refs. 34 and 38; see “Discussion”). However, to date, for mestranol and EE, no studies have been reported where their enhancing activities have been directly compared, where dose-response effects were analyzed, and where HC incidence was definitively determined. The present report describes the results of studies designed to address these issues. In addition, since corticosteroids are growth inhibitory for liver (9, 18), the effect of addition of β-methasone to a mestranol-containing diet was also determined. Two- and 3-dimensional analyses of GGT lesion number and size were performed as well as the histopathological evaluation of advanced lesions. The results clearly show the strong enhancing ability of EE and 2 doses of mestranol but not estradiol on GGT lesion number and HC incidence.

MATERIALS AND METHODS

Animal Treatment. Female Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) obtained in 2 groups were housed individually in wire-bottomed cages under controlled conditions of temperature, humidity, and lighting. The 12-month experiment was started 2 weeks prior to the 9-month experiment. Food and distilled water were available ad libitum. Upon arrival, the animals were fed a...
defined semisynthetic basal control diet (43) obtained from Teklad (Madison, WI). At approximately 7 weeks of age, the rats were subjected to a surgical two-thirds partial hepatectomy; 24 hr later, they were given i.p. injections of either 0.85% NaCl solution (noninitiated, -DEN groups) or DEN at 20 mg/kg body weight (initiated, +DEN groups). Twenty-four hr later, the animals were transferred (indicated by ➔) to the various treatment groups shown in Table 1.

The estrogens (Sigma Chemical Co., St. Louis, MO) were added to the basal diet as described previously (43), and the diet was subsequently fed in powder form. Mestranol was fed at 2 dose levels with the low dose [M(U)] being 0.1 ppm and the high dose [M(H)] being 0.5 ppm which represents approximately 3 and 15 times the human dose, respectively. On the low-dose diet, rats ingested approximately 6 μg mestranol/kg/day; at the high dose, they ingested 30 μg mestranol/kg/day. The concentration of ethinyl estradiol was 0.5 ppm, and that of mestranol/kg/day; at the high dose, they ingested 30 μg mestranol/kg/day. The presence of ethinyl estradiol was also present at 0.5 ppm, but the animals failed to gain weight and suffered hair loss. After 2 weeks, the dose of β-methasone was reduced to 0.2 ppm, and it remained at that level for the duration of the experiment. Food consumption and body weight data were obtained throughout the course of the experiment.

Histochemical and Histopathological Analyses. At the time of kill, the livers were removed and weighed, and a liver sample from 2 of the remaining 4 lobes was obtained by the use of a 15-mm diameter cork borer. This procedure4 provides samples of similar size from each lobe and each rat. The liver samples were frozen on dry ice and processed for GGT histochemistry as described previously (43) to reveal the presence of putative preneoplastic and neoplastic lesions. The GGT lesions detected consisted of foci and more advanced nodules and carcinomas. No attempt was made to histopathologically identify and evaluate the GGT lesions in H & E-stained serial sections. All GGT lesions scored were well defined, with the majority of cells being stained. In addition, our previous studies indicated that the synthetic estrogens induced the appearance of putative preneoplastic and neoplastic lesions. The GGT lesions detected consisted of foci and more advanced nodules and carcinomas. Care was taken to ensure that the diffuse staining was not scored as a GGT lesion.

A second sample was taken from the large right lobe for histopathological analysis, and the remaining liver was then sliced at 1- to 2-mm intervals to detect nodules which, if present, were also sampled. These samples were fixed and processed using paraffin embedding and H & E staining. Histopathological evaluation using the criteria described by Squier and Levitt (31) was undertaken without knowledge of treatment. The presence of nodules (preneoplastic nodules) and HCs was recorded. While these lesions commonly coexisted in the same liver, in Table 2 the lesions were present in the H & E-stained liver sections of all DEN-treated rats, but these were not counted.

GGT Lesion Quantitation. Numbers and areas of the GGT lesion transsections in one cryostat section from each liver sample (2 samples/rat) were determined using a Zeiss microscope in combination with a MOP-30 Digital Analyzer System (Carl Zeiss, Inc., Thornwood, NY). The smallest group of cells scored as a focus had a diameter of 50 μm. The area of tissue on each slide was determined by projecting the slide onto the calibrated MOP-30 digitizing board and tracing the section with a cursor. The 2-dimensional data representing GGT lesion transsection number and size were subjected to calculations of quantitative stereology using the equation for truncated data reported by Pugh et al. (23, 24). This allows representation of the data in 3-dimensional space when the experimenter is unable to reliably identify 2-dimensional transsections below a certain size limit. The size distribution of the lesions was calculated using the method of Saltykov (26, 27) as described by Campbell et al. (5). The smallest size class has been designated Size Class 1, and the smallest lesion transsection reproductively definable within this size class had a diameter of 50 μm. The size classes are defined by a log scale of maximum diameters (μm) by the series: Size Class 1, antilog 1.8; Size Class 2, antilog 1.9; Size Class 3, antilog 2.0 … Size Class 20, antilog 3.7, as originally described by Campbell et al. (5). In this size classification system, lesions in each subsequent size class have a volume 2 times greater than those in the previous size class, thus reflecting the increased tumor burden to the rat. The stereological calculations were not corrected for potential artifacts due to stretching and shrinkage. The GGT lesion transsections were essentially circular, thus representing essentially spherical growths. There was no evidence of distortion in an elliptical fashion as a result of the histological procedure.

Statistical Analyses of the Data. The data were subjected to a one-way analysis of variance with multiple comparisons among groups performed using the Neuman-Keuls procedure (2). Results reported as significant using this procedure are less than the 5% level of significance. In addition, in selected instances, 2 groups were compared using a 2-tailed t test. Nodule and HC incidence data were analyzed using the χ² test.

RESULTS

Effects of Treatment on Animal Growth. Chart 1 shows the body weight curves for the various groups of animals. In the 9-
### Table 1
Liver weight, tissue area examined, GGT lesion transsections per sq cm tissue, and 3-dimensional calculations for animals killed after 9 and 12 months

<table>
<thead>
<tr>
<th>Group*</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Liver wt (g)</th>
<th>Area of tissue (sq cm)</th>
<th>Lesion transsections/ sq cm</th>
<th>Lesions/cu cm</th>
<th>Lesions/liver</th>
<th>Mean lesion diameter (μm)</th>
<th>Mean lesion volume (cu mm)</th>
<th>Lesion volume as % of liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>SAL → basal</td>
<td>12</td>
<td>11.0 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.99</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.172 ± 0.156</td>
</tr>
<tr>
<td>2</td>
<td>SAL → estradiol</td>
<td>12</td>
<td>11.4 ± 0.6</td>
<td>2.92</td>
<td>0.47 ± 0.27</td>
<td>14 ± 7&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;</td>
<td>136 ± 64</td>
<td>313 ± 85</td>
<td>0.172 ± 0.156</td>
<td>0.31 ± 0.29</td>
</tr>
<tr>
<td>3</td>
<td>SAL → EE</td>
<td>12</td>
<td>8.4 ± 0.3</td>
<td>2.86</td>
<td>5.25 ± 0.96&lt;sup&gt;c&lt;/sup&gt;</td>
<td>148 ± 28&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1249 ± 240&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>366 ± 18</td>
<td>0.031 ± 0.004</td>
<td>0.48 ± 0.11</td>
</tr>
<tr>
<td>4</td>
<td>SAL → M(H)</td>
<td>12</td>
<td>10.1 ± 0.3</td>
<td>2.88</td>
<td>4.17 ± 1.15&lt;sup&gt;e&lt;/sup&gt;</td>
<td>122 ± 30&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1193 ± 598&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>379 ± 46</td>
<td>0.046 ± 0.013</td>
<td>0.50 ± 0.22</td>
</tr>
<tr>
<td>5</td>
<td>+DEN → basal</td>
<td>11</td>
<td>11.2 ± 0.3</td>
<td>3.04</td>
<td>2.27 ± 1.15</td>
<td>85 ± 42</td>
<td>1116 ± 643</td>
<td>279 ± 19</td>
<td>0.013 ± 0.003</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>+DEN → estradiol</td>
<td>13</td>
<td>11.5 ± 0.8</td>
<td>2.89</td>
<td>1.19 ± 0.35</td>
<td>43 ± 13</td>
<td>461 ± 125</td>
<td>306 ± 39</td>
<td>0.120 ± 0.106</td>
<td>0.16 ± 0.11</td>
</tr>
<tr>
<td>7</td>
<td>+DEN → EE</td>
<td>13</td>
<td>9.0 ± 0.6</td>
<td>2.88</td>
<td>15.40 ± 1.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>446 ± 46</td>
<td>3879 ± 398&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>354 ± 18&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.049 ± 0.011&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.98 ± 0.29</td>
</tr>
<tr>
<td>8</td>
<td>+DEN → M(H)</td>
<td>13</td>
<td>9.8 ± 0.2</td>
<td>2.78</td>
<td>14.98 ± 1.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>506 ± 57</td>
<td>4899 ± 531</td>
<td>323 ± 31</td>
<td>0.225 ± 0.185</td>
<td>5.19 ± 3.25&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>+DEN → M(H) + β-methasone</td>
<td>10</td>
<td>10.6 ± 0.8</td>
<td>2.83</td>
<td>10.68 ± 1.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>295 ± 43&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3163 ± 487&lt;sup&gt;e&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>387 ± 30</td>
<td>0.157 ± 0.058</td>
<td>4.09 ± 1.57</td>
</tr>
</tbody>
</table>

| 12 months |                           |             |              |                        |                            |                |              |                          |                            |                             |
| 10      | SAL → basal               | 16          | 14.3 ± 1.1  | 2.59                   | 2.25 ± 0.66<sup>d</sup>,<sup>i</sup> | 101 ± 29<sup>d</sup>,<sup>i</sup> | 1234 ± 376<sup>d</sup>,<sup>i</sup> | 281 ± 30                   | 0.020 ± 0.004              | 0.11 ± 0.04                |
| 11      | SAL → M(L)                | 16          | 11.3 ± 0.5  | 2.55                   | 5.38 ± 1.24<sup>c</sup>,<sup>h</sup> | 155 ± 37       | 1518 ± 344<sup>c</sup> | 378 ± 30<sup>e</sup>               | 0.052 ± 0.012              | 0.74 ± 0.21<sup>c</sup>,<sup>h</sup> |
| 12      | SAL → M(H)                | 16          | 10.5 ± 0.5  | 2.53                   | 3.88 ± 1.24<sup>c</sup>,<sup>h</sup> | 155 ± 37       | 1518 ± 344<sup>c</sup> | 378 ± 30<sup>e</sup>               | 0.052 ± 0.012              | 0.74 ± 0.21<sup>c</sup>,<sup>h</sup> |
| 13      | +DEN → basal              | 16          | 12.7 ± 0.7  | 2.53                   | 1.92 ± 0.49<sup>g</sup>,<sup>i</sup> | 57 ± 13<sup>g</sup>,<sup>i</sup> | 720 ± 153    | 353 ± 37                  | 0.102 ± 0.057              | 0.47 ± 0.22                 |
| 14      | +DEN → M(L)               | 17          | 9.4 ± 0.5   | 2.51                   | 8.10 ± 1.41<sup>d</sup> | 285 ± 52<sup>c</sup>,<sup>h</sup> | 2762 ± 464<sup>c</sup>,<sup>h</sup> | 297 ± 19                   | 0.031 ± 0.006              | 0.96 ± 0.17                 |
| 15      | +DEN → M(H)               | 15          | 12.4 ± 0.9  | 2.49                   | 15.63 ± 1.64<sup>c</sup>,<sup>h</sup> | 454 ± 38<sup>m</sup> | 5730 ± 763<sup>m</sup> | 342 ± 14                   | 0.086 ± 0.020              | 3.85 ± 0.79<sup>m</sup> |

* Group numbers are assigned to treatment groups for statistical comparisons and do not necessarily correlate with those in Table 2.

<sup>a</sup> Mean ± S.E.

<sup>b</sup> Significantly greater than Group 1, p < 0.05.

<sup>c</sup> Significantly greater than Group 2, p < 0.05.

<sup>d</sup> Significantly greater than Group 3, p < 0.05.

<sup>e</sup> Significantly greater than Group 4, p < 0.05.

<sup>f</sup> Significantly less than Group 8, p < 0.05.

<sup>g</sup> This value becomes 0.041 ± 0.017 when one rat (Rat 10) having very many large lesions such that its mean lesion volume was 2.4323 is omitted.

<sup>h</sup> This value becomes 2.03 ± 0.84 when one rat (Rat 10) having very many large lesions such that its lesion volume as percentage of liver was 43.1 is omitted. Group 8 is then significantly greater than Group 5.

<sup>i</sup> Significantly greater than Group 10, p < 0.05.

<sup>j</sup> Significantly greater than Group 11, p < 0.05.

<sup>k</sup> Significantly greater than Group 13, p < 0.05.

<sup>l</sup> Significantly greater than Group 14, p < 0.05.
month experiment (Chart 1A), all animals ingesting diet containing either mestranol or EE exhibited a decreased rate of weight gain compared to animals on basal diet or diet containing 17β-estradiol. Initiation with DEN did not influence subsequent body weight gain. At the time of kill, body weights of animals in groups ingesting mestranol or EE were significantly less that those in other groups. Similar results were observed in the 12-month experiment (Chart 1B) where, at the time of kill, the body weights of animals in the M(L) and M(H) groups were significantly less than those for the animals fed basal diet. The difference between the M(L) and M(H) groups was not significant.

Significant differences among groups in actual liver weights were not evident (Table 1). However, at 9 months, liver weight per 100 g body weight was significantly elevated in all groups being fed diet containing mestranol or EE compared to groups fed basal diet (data not shown). Estradiol feeding did not cause an increase in this parameter. At 12 months, animals fed M(H) exhibited a significant increase in liver weight per 100 g body weight compared to the groups fed basal diet (data not shown). Similar effects of mestranol on relative liver size were observed previously (43).

GGT Lesion Number and Size in Noninitiated Rats at 9 Months. Table 1 shows the data obtained from quantitative 2- and 3-dimensional analyses of the GGT lesions. At 9 months, no GGT lesions were detected in noninitiated (SAL) rats fed the control, basal diet. Estradiol caused the appearance of GGT lesions in only 4 of 12 noninitiated rats. In contrast, the number of GGT lesions was dramatically increased to comparable levels in noninitiated rats fed diet containing EE and mestranol. These increases were apparent when the data were expressed in 2 dimensions, as lesion transactions per sq cm, and in 3 dimensions, as lesions per cu cm or lesions per liver. No significant differences in mean lesion diameter or mean lesion volume were seen in noninitiated rats.

GGT Lesion Number and Size in Initiated Rats at 9 Months. Initiated rats subsequently fed basal diet developed low numbers of GGT lesions (Table 1). Feeding DEN-initiated rats diet containing EE or mestranol significantly enhanced the number of such lesions to levels greater than the sum of those seen in noninitiated animals fed these estrogens plus those initiated and fed basal diet. When the data are expressed as lesions per liver, the increase caused by EE was 3.5 times and the increase caused by mestranol was 4.4 times the number of lesions observed in initiated rats fed basal diet. It should be noted that this most probably represents an underestimate of the fold increase caused by EE and mestranol because at 9 months in the DEN → basal group, one rat of the 11 had an extraordinarily high (8182) number of GGT lesions. If this rat is dropped, the number of lesions per liver in this control group becomes 479 ± 99, and the increase by EE and mestranol becomes 8- and 10-fold, respectively. Estradiol did not cause an increase in the number of GGT lesions over that seen in the initiated controls. Table 1 also shows that the number of GGT lesions in the M(H) plus β-methasone group was significantly less than in the M(H) group.

The size of the GGT lesions is represented by both the mean lesion diameters and the mean lesion volumes. In initiated rats, the mean lesion size in the group fed EE was significantly greater than in the control group fed basal diet (Table 1). Chart 2 shows the size distribution of GGT lesions in the various treatment groups at 9 months. The 20 different size classes were determined as described by Campbell et al. (5) with the smallest group of cells scored being represented by Size Class 1. While, with the exception of the initiated rats fed EE, the mean lesion sizes among groups are not different (Table 1), the data represented in Chart 2 show that more large GGT lesions are present in the initiated rats fed EE or mestranol than in the initiated, basal diet groups or in their respective noninitiated (−DEN) control groups. The increased volume occupied by the lesions expressed as a percentage of the liver (Table 1) is due to the presence of many more lesions and an increase in the volume occupied by large lesions (Chart 2, bottom).

GGT Lesion Number and Size in Noninitiated Rats at 12 Months. Table 1 also shows data from the longer-term 12-month experiment concerned with establishing a mestranol dose re-
response. Here again, no GGT lesions were detected in noninitiated rats fed basal diet. In noninitiated (SAL) rats, mestranol at the low [M(L)] and high [M(H)] doses caused a large increase in the number of GGT lesions compared to controls fed basal diet. When the data are represented in 2 dimensions as lesions/sq cm, the difference in GGT lesion transection number between the M(L) and M(H) dose groups is significant. However, following transformation to 3 dimensions, the difference between doses loses significance at the 5% level. If more lesion transections are detected in the 2-dimensional tissue section but not in the 3-dimensional tissue, then it follows that the lesions must generally be larger, thus accounting for the greater probability that their transections will be detected in the 2-dimensional analysis. This point is illustrated in Table 1 where the mean lesion diameter of the M(H) group is significantly greater than that of the M(L) group. This can also be seen in Chart 3 where the number of lesions in each size class is shown for the 12-month experiment. A greater number of larger lesions is present in the noninitiated (-DEN) animals fed M(H) than in those fed M(L).

**GGT Lesion Number and Size in Initiated Rats at 12 Months.**
In the DEN-initiated rats, the main effect of mestranol was to cause a dramatic dose-dependent enhancement in the number of GGT lesions. The differences among the basal, M(L), and M(H) groups were significant. When the data are expressed as GGT lesions per liver, it can be seen that M(L) caused a 4-fold increase over the control group and M(H) caused a 2-fold increase over the M(L) group. Comparison of the 9- and 12-month data among the DEN-initiated rats reveals no significant increase with time in the number of GGT lesions per liver in the groups fed basal diet (controls) or M(H).

With regard to GGT lesion size in the initiated, 12-month animals, Table 1 shows that no significant increase in mean lesion diameter or volume with increased mestranol dose was detected. However, close examination of the size distributions of the GGT lesions as shown in Chart 3 shows the presence of more large lesions in the M(H) group. Thus, while the mean lesion size does not appear to increase with mestranol dose, more larger lesions are present. Further support for the notion that promotion by mestranol results in a dose-dependent increase in both the number of lesions and the appearance of more larger lesions comes from the data shown in Chart 4. Here, the data are presented as number of lesions per cu cm (left ordinate) and number of lesion transections per sq cm (right ordinate). As shown previously by Campbell et al. (5), when the size distributions between treatment groups of the GGT lesions are similar, the data must show proportional dose relationships when expressed in 2 dimensions (lesion transections/sq cm) and 3 dimensions (lesions/cu cm). The data in Chart 4 do not show proportional dose-response relationships indicating that the mean diameters of the lesions in the 3-dimensional liver are not similar between these treatment groups. Thus, overall, the 12-month data show that mestranol causes a dose-dependent increase in GGT lesion number and the appearance of larger lesions.

**Effect of Treatment on Liver Nodule and Carcinoma Incidence.**
The data in Table 2 show the incidence of liver nodules and HC as a function of treatment in the 9- and 12-month animals. At 9 months, no nodules or carcinomas were detected in the noninitiated (SAL) animals. In DEN-initiated rats, the incidences of HC and nodules plus HC combined were significantly increased compared to control in animals treated with EE and mestranol but not estradiol. While addition of $\beta$-methasone to the mestranol-containing diet caused a significant decrease in the number of GGT lesions (Table 1), the incidence of HC was not reduced. In fact, the incidence of HC in that group was significantly higher than in the M(H) group. These results clearly show that both synthetic estrogens enhance the incidence of HC after 9 months of treatment.

At 12 months, in the noninitiated animals, the M(H) group exhibited a significant increase in the combined incidence of nodules plus HC. In DEN-initiated animals, the incidence of HC was significantly increased in the M(H) group. In the M(L) group, the incidence of HC was somewhat higher than in the control group, but the difference was not significant.
However, in light of results reported by others where the results obtained in the 12-month study demonstrate that, in the conditions of this study, did not act as a promoter. Secondly, the appearance of either GGT lesions or HC and therefore, under genic effects under certain protocols. Estradici did not enhance ranging from 1 to 4 weeks following initiation (3, 4, 32-34), it is that the start of synthetic estrogen treatment was delayed for times observed could be due to a cocarcinogenic effect of these agents treatment with the synthetic estrogens was started 24 hr after carcinogenesis. First, this study clearly demonstrates that by 9 months both EE and mestranol exert comparable enhancing treatment with synthetic estrogens in hepatocarcinogenesis. First, this study clearly demonstrates that by 9 months both EE and mestranol exert comparable enhancing effects on the number of GGT-positive lesions and on the incidence of HC in DEN-initiated rats. In this and our previous study, treatment with the synthetic estrogens was started 24 hr after DEN initiation. Thus, the enhancement of hepatocarcinogenesis observed could be due to a cocarcinogenic effect of these agents (37, 38). However, in light of results reported by others where the start of synthetic estrogen treatment was delayed for times ranging from 1 to 4 weeks following initiation (3, 4, 32-34), it is likely that EE and mestranol can be regarded as promoters of this notion that the effects of the synthetic estrogens are largely hepatocarcinogenesis. The results obtained indicated that enhancement of hepatocarcinogenesis by these agents does result in the appearance of larger lesions. However, their size distribution was not affected to the extent observed with PB and TCDD, even though these agents have similar effects on the numbers of GGT lesions induced compared to their respective controls. More comprehensive comparative dose-time studies are required to define the influence of various enhancers of hepatocarcinogenesis on the growth rate of putative preneoplastic lesions in the liver (21).

**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats/group</th>
<th>Nodules</th>
<th>HC</th>
<th>Combined</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>SAL → basal</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>SAL → estradiol</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>SAL → EE</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>SAL → M(H)</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>SAL → M(L)</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>+DEN → basal</td>
<td>13</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>+DEN → estradiol</td>
<td>13</td>
<td>4</td>
<td>6</td>
<td>10*</td>
</tr>
<tr>
<td>8</td>
<td>+DEN → EE</td>
<td>13</td>
<td>4</td>
<td>5</td>
<td>9*</td>
</tr>
<tr>
<td>9</td>
<td>+DEN → M(H)</td>
<td>13</td>
<td>4</td>
<td>5</td>
<td>9*</td>
</tr>
<tr>
<td>10</td>
<td>+DEN → M(L)</td>
<td>13</td>
<td>4</td>
<td>5</td>
<td>9*</td>
</tr>
<tr>
<td>11</td>
<td>+DEN → M(H) + β-methasone</td>
<td>9</td>
<td>1</td>
<td>8*</td>
<td>9*</td>
</tr>
</tbody>
</table>

* Group numbers are assigned to treatment groups for statistical comparisons and do not necessarily correlate with those in Table 1.

**DISCUSSION**

The results of the present study confirm and extend our previous findings (39, 40, 43) and the recent work of others (4, 5, 32-34) regarding the role of synthetic estrogens in hepatocarcinogenesis. First, this study clearly demonstrates that by 9 months both EE and mestranol exert comparable enhancing effects on the number of GGT-positive lesions and on the incidence of HC in DEN-initiated rats. In this and our previous study, treatment with the synthetic estrogens was started 24 hr after DEN initiation. Thus, the enhancement of hepatocarcinogenesis observed could be due to a cocarcinogenic effect of these agents (37, 38). However, in light of results reported by others where the start of synthetic estrogen treatment was delayed for times ranging from 1 to 4 weeks following initiation (3, 4, 32-34), it is likely that EE and mestranol can be regarded as promoters of hepatocarcinogenesis, although they may also exert cocarcinogenic effects under certain protocols. Estradiol did not enhance the appearance of either GGT lesions or HC and therefore, under the conditions of this study, did not act as a promoter. Secondly, the results obtained in the 12-month study demonstrate that, in DEN-initiated rats, the increase in GGT lesion number follows a clear dose-response relationship to mestranol. However, a significant increase in incidence of HC was observed only in the M(H) group.

While the present study was not designed to provide detailed information regarding the effects of these synthetic estrogens on the growth rate of GGT lesions, we did obtain information on the size distribution of these putative preneoplastic lesions in animals from various treatment groups. The effects of the synthetic estrogens on the size distribution of the GGT lesions do not appear to be as dramatic as those observed by Campbell et al. (5) for PB and by Pitot et al. (22) for TCDD. In the former study (5), 500 ppm dietary PB caused a 3.8-fold increase in the number of GGT foci per cu cm, a 2.1-fold increase in their mean diameters, and an 11.8-fold increase in their mean volume compared to non-PB-treated controls. In the latter study (22), TCDD at the high dose (1.4 μg/kg, 14 twice-weekly injections) caused a 2.8-fold increase in the number of GGT foci per cu cm and a 24.5-fold increase in their mean volume. In the present study, we saw that, at 9 months, mestranol at 0.5 ppm in the diet caused at least a 5.9-fold increase in GGT lesions/cu cm but no significant changes in either mean lesion diameter or mean lesion volume. At 12 months, 0.5 ppm mestranol caused an 8-fold increase in lesions per cu cm, but again no significant increase in mean diameter or volume was detected. These data support the notion that the effects of the synthetic estrogens are largely on the number of GGT lesions. It is also apparent from the charts showing the numbers of lesions in the various size classes that enhancement by these agents does result in the appearance of larger lesions. However, their size distribution was not affected to the extent observed with PB and TCDD, even though these agents have similar effects on the numbers of GGT lesions induced compared to their respective controls. More comprehensive comparative dose-time studies are required to define the influence of various enhancers of hepatocarcinogenesis on the growth rate of putative preneoplastic lesions in the liver (21).

Studies by Loeb (18) and Desser-Wiest (9) have demonstrated that corticosteroids can be inhibitory for liver growth. In light of these findings, we attempted to determine whether the glucocorticoid β-methasone would have an effect on the enhancement of hepatocarcinogenesis. The results obtained indicated that addition of β-methasone to the mestranol-containing diet did cause a significant reduction in GGT lesion number. However, the incidence of HC in this group was 90% which is significantly greater than that in the M(H) group itself. One possible explanation for this result is that, as indicated by the rat growth curves, the combination of M(H) and β-methasone had a somewhat greater toxic effect than did mestranol alone. If this reflects increased hepatotoxicity (see below) which may be an important component of promotion by at least some agents, it may at least partially account for the increased incidence of HC in this group.

At this point, the mechanism by which the synthetic estrogens enhance hepatocarcinogenesis through promotion is unknown. It is apparent from the body weight data that these agents, even at the low dose levels used in this study, exert toxic effects on the animals. In our previous study (43), in the present study, and in on-going experiments, we observed that both synthetic estrogens caused the appearance of diffuse GGT staining radiating from the portal areas. No distinct cellular alterations were ob-
erved in the H & E-stained sections; except in one or 2 in-
estances, this staining did not complicate scoring of GGT foci. 
Determination of GGT activity in liver homogenates confirmed 
the presence of elevated levels of this enzyme in the synthetic 
estrogen-treated rats (43). Studies by others have indicated that 
increased liver GGT is associated with intrahepatic cholestasis 
in the absence of ductal cell proliferation (25). Cholestasis has 
been reported to occur in rats treated with various synthetic 
estrogens (7, 8), and it may be that this type of chronic toxic 
effect contributes to or is responsible for their promoting activity. 
The synthetic estrogens have been shown to stimulate liver cell 
proliferation (6). This response may be due to mitogenic effects 
on the liver and/or may be a manifestation of restorative hyper-
plasia in response to chronic toxicity. Two recent reports have 
presented the results of studies which demonstrate that feeding 
a choline-deficient diet causes hepatocyte toxicity as detected 
by the loss of [3H]thymidine from prelabeled liver DNA (12, 13). 
In the report by Gianbarrasi et al. (13), it was also demonstrated 
that this loss of prelabel was accompanied by a stimulation of 
liver cell proliferation. It was suggested in both reports that 
the restorative hyperplasia has a role in the ability of the choline-
deficient diets to promote hepatocarcinogenesis. One might 
predict that promotion by the synthetic estrogens involves similar 
mechanisms, and their effects on prelabeled liver DNA stability 
and new synthesis (42) are currently under investigation in our 

A second possible mechanism of promotion by the synthetic 
estrogens may directly or indirectly involve their estrogenic activ-
ity. This possibility is not supported by the finding that, under 
the conditions of this study, estradiol did not enhance the ap-
pearance of GGT lesions or HC. This may indicate that the 
natural estrogen is not a promoter and that promotion by the 
synthetic estrogens is a result of their toxic effects. However, it 
is also possible that estradiol itself was rapidly metabolized by 
the liver, thus preventing elevated levels from accumulating. 

In the present study, we did not observe any GGT lesions in 
the noninitiated rats fed basal diet. At first glance, this may seem 
to be in contrast to the results observed by others (20, 28, 29, 
35). However, our study was of shorter duration than most of 
the others and in addition differed from them in one or more of 
the following ways: rat strain, sex, diet composition, or pheno-
type of the foci being scored. Thus, comparisons of the sponta-
neous incidences and small numbers of foci among these studies 
do not seem appropriate.

As reported previously (39, 40, 43) and as seen in this study, 
mestranol or EE treatment of noninitiated rats enhanced the 
appearance of GGT lesions and nodules plus HC. This response 
could occur if these agents possessed weak initiating activity 
which, coupled with their promoting activity, rendered them weak 
"complete carcinogens." Alternatively, the synthetic estrogens, 
being relatively strong promoters, may be promoting the ap-
pearance of liver lesions arising from spontaneously initiated 
hepatocytes of the type recently reported by Schulte-Hermann 
et al. (29). Various studies reported by several laboratories 
suggest that the synthetic estrogens lack genotoxic potential 
and thus are not initiating agents. For example, while EE can be 
metabolized to reactive intermediates capable of binding to DNA, 
the level of binding is 10,000 times less than that of known 
hepatocarcinogens (16). Furthermore, the synthetic estrogens 
are not mutagenic in bacterial (17) or mammalian (10) cell muta-
genicity assays, nor do they appear to cause chromosomal 
aberrations in bone marrow cells in vivo (1). We recently reported 
that the synthetic estrogens do not cause detectable levels of 
DNA damage in liver in vivo or in isolated hepatocytes and that 
mestranol does not exhibit strong initiating potential for liver (41). 
In addition, Schuppert et al. (30) recently reported several endo-
genous and synthetic estrogens, including EE, to be negative for 
tumor-initiating activity in liver.

The present study provides additional support for the notion 
that the synthetic estrogens lack initiating potential and thus are 
not complete carcinogens. There are at least 2 criteria that 

promoters not possessing initiating activity must meet in addition 
to the lack of genotoxic activity. These are: (a) upon chronic 
treatment of noninitiated animals, tumorigenesis should exhibit 
a plateau with regard to promoter dose; and (b) at a maximum 
dose of promoter, tumorigenesis should exhibit a plateau with 
respect to time. In other words, tumor promoters should exhibit 
effects lacking summation with dose and time on tumorigenesis. 
Examination of the data for noninitiated animals presented in 
Table 1 and Chart 4 in the present report reveals that mestranol 
meets these criteria. Mestranol does not cause an increase in 
GGT lesion number between 9 and 12 months and does not 
cause a significant increase in GGT lesions per liver with in-
creased dose. These facts argue against the synthetic estrogens 
having complete carcinogenic activity.

In summary, the results presented in this report demonstrate 
that the synthetic estrogens, mestranol and EE, dramatically 
enhanced the number of GGT lesions detected and the incidence 
of hepatocellular carcinomas and caused an increase in the 
number of GGT lesions in the larger size classes. These results 
together with those reported by others and discussed above 
suggest that this enhancement occurs by promotion of hepato-
carcinogenesis.

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Promotion by Synthetic Estrogens


Enhancement of Hepatocarcinogenesis in Female Rats by Ethinyl Estradiol and Mestranol but not Estradiol

James D. Yager, Harold A. Campbell, Daniel S. Longnecker, et al.