Biochemical Effects of the Mouse Hepatocarcinogen Oxazepam: Similarities to Phenobarbital

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The benzodiazepines are one of the most commonly used classes of drugs. Some of the better known benzodiazepines include diazepam (Valium), chlordiazepoxide (Librium or Libritabs), and oxazepam (Serax). They are used primarily as sedative-hypnotics and antianxiety agents, and to a lesser extent as muscle relaxants and anticonvulsants (Harvey, 1985). Oxazepam is also a common metabolite of many other benzodiazepines including both diazepam and chlordiazepoxide (Langner et al., 1991).

In a recent study, the National Toxicology Program determined oxazepam to be a mouse liver carcinogen (NTP, 1993; Bucher et al., 1994). Increased incidences of hepatocellular adenomas and carcinomas were detected in the high-dose groups (2500 and 5000 ppm) in both sexes, but not at 125 ppm. Increased incidences of thyroid hyperplasia in male and female B6C3F1 mice and follicular cell adenomas in females were also dose related. The relevance of oxazepam-mediated carcinogenicity in mice to humans is as yet unknown. Therefore, a mechanism-based evaluation of the risk to man would provide the best approach for the regulation of oxazepam and the other benzodiazepines which are metabolized to oxazepam.

A series of studies was therefore initiated to address the mechanism of oxazepam-induced carcinogenicity in mice. Earlier studies showed that following a single dose the mouse is a poor model for human metabolism of oxazepam. However, after repeated exposure in dosed feed studies, mouse metabolism more closely resembles that in man (Griffin and Burka, 1993). This was apparently the result of induction of drug metabolizing enzymes. These same studies provided little evidence that reactive metabolites were generated. Another study detected a brief but intense burst of hepatocellular proliferation shortly after the start of dosed feed treatment in mice (Cunningham et al., 1994). Transient proliferation of this sort is not considered to be sufficient to evoke a tumorigenic response of the magnitude seen in the NTP study (Ledda-Columbano et al., 1989). Therefore, the proliferative response probably contributes to the carcinogenicity only in conjunction with another factor.

In this investigation, we evaluated the early time course of oxazepam-induced cell proliferation and examined some of the other possible mechanisms that may contribute to the induction of mouse hepatocarcinogenesis in concert with cell proliferation. Although it is likely that oxazepam is not a genotoxic agent, a genotoxic effect has been reported under some conditions (Batzinger et al., 1978; NTP, 1993; Stopper et al., 1993). Therefore, we examined the possible involvement of free radicals and glutathione (GSH) depletion resulting in increased hepatocyte susceptibility to oxidative stress. Multiple similarities between oxazepam and phenobarbital suggested another approach. Both drugs are anxiolytics, tumor promoters, mouse liver carcinogens, liver mitogens, and phenobarbital-type enzyme inducers (Bucher et al., 1994; Griffin et al., 1995; Rice et al., 1994; Sasaki et al., 1983). The mechanism of phenobarbital-induced thyroid carcinogenesis in rats is believed to be via the alteration...
of circulating thyroid hormone levels secondary to enzyme induction (McClain, 1989). Therefore, we examined oxazepam-induced changes in thyroid-stimulating hormone (TSH) and expanded our earlier investigation of oxazepam-mediated hepatic enzyme induction (Griffin et al., 1995).

MATERIALS AND METHODS

Animals. Male B6C3F1 mice (18–22 g) were obtained from Charles River Breeding Laboratories (Laleigh, NC). Mice were randomly assigned to cages (five per cage) and allowed to acclimatize to animal facility conditions and to powder feed dispensed from stainless-steel open box dispensers for at least 2 weeks.

Treatments. Mice were randomly divided into 12 groups (enzyme activities, N = 15/group, 3 cages/group; cell proliferation, N = 5/group) for treatment with dosed feed. Animals received powdered dosed feed containing oxazepam (Roussel Corporation, Englewood Cliffs, NJ) at doses of 0, 125, and 2500 ppm for 3-, 7-, 10-, or 21-day treatment periods. The start of treatments was staggered so that all treatments were concluded with a 4-day period and mice were therefore the same age ± 4 days at the time of euthanization.

Sample preparation. Animals were euthanized with carbon dioxide and weighed. Livers were rapidly removed, weighed, and placed in ice-cold Tris/KCl buffer (20 mM Tris base, 150 mM KCl, 1 mM EDTA, pH 7.4). Livers were pooled (3–4/N, N = 4), minced with scissors, and homogenized with three strokes of a motorized Teflon pestle in a glass mortar. Homogenates were centrifuged at 2500g for 20 min and then the pellet was discarded. The supernatant was then frozen at −80°C until needed. Microsomes and cytosol were later prepared from aliquots of homogenate by standard methods.

Equipment. The determination of superoxide dismutase, cytochrome P450, and cytochrome b5 were made on a Beckman DU-8 spectrophotometer (SLM Instruments, Urbana, IL). All other determinations were performed on a Beckman DU-8 spectrophotometer.

Cell proliferation. Hepatic cell proliferation by evaluation of Brdu incorporation into newly replicated cellular DNA was performed as described earlier (Cunningham et al., 1994). All animals were exposed to Brdu for 7 continuous days prior to euthanization via an osmotic mini-pump (Alzet Model 2002, Palo Alto, CA).

Serum TSH. Immediately following CO2 euthanization, blood was collected from the retroorbital sinus of cell proliferation animals. The blood was allowed to clot and then centrifuged at 1500 rpm for 10 min. TSH levels in the plasma were quantitated with an enzyme-linked immunosassay kit (Sigma Chemical Co.).

Determination of homogenate enzyme contents and activities. Contents of cytochromes P450 and b5 in mouse liver homogenates were determined by difference spectral methods (Omura and Sato, 1964). Control values were consistent with reported literature activities (Sasaki et al., 1991; Slater and Sawyer, 1971). Nonprotein sulfhydryl (NPS) and thiobarbituric acid reactive products were measured according to established methods (Standeven and Wetterhahn, 1991; Griffin et al., 1995). Microsomal protein content (mg of microsomal protein/g of liver) was also determined in a time- and dose-dependent manner by oxazepam treatment. Cytosolic protein content (mg of cytosolic protein/g of liver) was not altered in a consistent manner by oxazepam treatment.

RESULTS

The effects of oxazepam-dosed feed treatment on liver-to-body weight ratios and microsomal and cytosolic protein contents are presented in Table 1. Liver-to-body weight ratios were elevated in a time- and dose-dependent manner precisely as has been reported previously (NTP, 1993; Cunningham et al., 1994; Griffin et al., 1995). Microsomal protein content (mg of microsomal protein/g of liver) was also elevated in a time- and dose-dependent manner by oxazepam treatment. Cytosolic protein content (mg of cytosolic protein/g of liver) was not altered in a consistent manner by oxazepam treatment.

Total P450 content expressed as per milligrams of homogenate per gram of liver and total activity per liver increased with time and dose of oxazepam. The increases were up to 3x that of control values (Table 2). The same response was detected for cytochrome b5 levels (Table 3).

At 10 days of treatment p-nitrophenol glucuronyl transferase activity was compared between 0- and 2500-ppm dosed feed. Activity in the high-dose group (235.0 ± 17.3 μmol of glucuronide generated/liver/min) was significantly elevated (p < 0.001) over that in controls (115.4 ± 10.6 μmol of glucuronide generated/liver/min).

In Table 4 the effects of oxazepam-dosed feed treatment on various indicators of free radical generation are presented. No dose-dependent elevations were detected in lipid peroxidation products. Differences between days in control lipid peroxidation and superoxide dismutase values were due to interassay variability (each time point was assayed at a different time and within assay differences were consistent and readily repeatable). Superoxide dismutase and glutathione peroxidase activities were also not elevated. Catalase activity was significantly elevated at 2500 ppm after 7 and 10 days of treatment.

The effects of oxazepam treatment on nonprotein sulfhydryl levels are presented in Fig. 1. At the high dose of 2500 ppm, catalase activity is not measured, but the values are presented in Table 1.

Abbreviations used: Brdu, bromodeoxyuridine; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; NPS, nonprotein sulfhydryl; TSH, thyroid-stimulating hormone.
ppm NPS was slightly reduced at 3, 7, 10, and 21 days. This response is statistically significant at 3 and 21 days of treatment.

Serum TSH levels were unaffected by treatment with oxazepam at 2500 ppm in the diet at 3 and 7 days. By 10 days of treatment, serum TSH levels rose to 300% of untreated controls and returned to control values by 21 days of continuous oxazepam treatment (Fig. 2). Serum TSH was unaffected by treatment at 125 ppm (data not shown).

Similarly, hepatic cell proliferation was unchanged by dietary treatment with 2500 ppm oxazepam after 3 and 7 days. By 10 days of treatment, the hepatic labeling index was significantly elevated compared to control. Hepatic labeling index was reduced by 21 days of oxazepam treatment compared to the levels observed at 10 days, but was still significantly elevated compared to control levels (Fig. 2). Exposure to 125 ppm oxazepam increased hepatic labeling by 7 days, with a return to control levels by 21 days. The earlier response at the lower dose was probably the result of less sedation and consequently greater food consumption than in the 2500-ppm group.

### DISCUSSION

In a series of earlier investigations the effects of oxazepam-dosed feed treatment on metabolism, cell proliferation,

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**TABLE 1**

Liver-to-Body Weight Ratios and Liver Cell Protein Composition Following Subchronic Treatment with Oxazepam-Dosed Feed in B6C3F1 Mice

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Dose (ppm)</th>
<th>Liver-to-body weight ratio</th>
<th>Microsomal protein content*</th>
<th>Cytosolic Protein content\†</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>0.056 ± 0.001</td>
<td>8.05 ± 0.83</td>
<td>58.37 ± 3.85</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>0.059 ± 0.001</td>
<td>6.72 ± 0.25</td>
<td>55.47 ± 0.87</td>
</tr>
<tr>
<td>3</td>
<td>2500</td>
<td>0.047 ± 0.001*</td>
<td>10.44 ± 0.27*</td>
<td>60.07 ± 9.17</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0.057 ± 0.001</td>
<td>12.04 ± 0.92</td>
<td>53.80 ± 4.21</td>
</tr>
<tr>
<td>7</td>
<td>125</td>
<td>0.062 ± 0.001*</td>
<td>11.40 ± 0.33</td>
<td>54.72 ± 2.12</td>
</tr>
<tr>
<td>7</td>
<td>2500</td>
<td>0.074 ± 0.002*</td>
<td>14.67 ± 0.21*</td>
<td>63.60 ± 1.98*</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0.056 ± 0.001</td>
<td>9.90 ± 0.79</td>
<td>60.32 ± 0.87</td>
</tr>
<tr>
<td>10</td>
<td>125</td>
<td>0.061 ± 0.001*</td>
<td>10.60 ± 0.24</td>
<td>52.20 ± 2.90*</td>
</tr>
<tr>
<td>10</td>
<td>2500</td>
<td>0.076 ± 0.001*</td>
<td>12.95 ± 0.50*</td>
<td>57.32 ± 2.00</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>0.053 ± 0.001</td>
<td>7.79 ± 0.49</td>
<td>62.12 ± 1.39</td>
</tr>
<tr>
<td>21</td>
<td>125</td>
<td>0.061 ± 0.001*</td>
<td>9.43 ± 0.92</td>
<td>58.92 ± 2.85</td>
</tr>
</tbody>
</table>

\* Milligrams of microsomal protein/gram of liver.

\† Milligrams of cytosolic protein/gram of liver.

\*° Significantly different from control at \*\((p < 0.05)\), \*\((p < 0.01)\), or \*\((p < 0.001)\).

**TABLE 2**

Induction of Cytochrome P450 by Subchronic Oxazepam-Dosed Feed Treatment in Male B6C3F1 Mice

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Dose (ppm)</th>
<th>nmol P450/mg homogenate</th>
<th>nmol P450/g liver</th>
<th>nmol P450/liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>0.236 ± 0.008</td>
<td>19.30 ± 1.91</td>
<td>28.84 ± 2.68</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>0.208 ± 0.016</td>
<td>16.39 ± 0.58*</td>
<td>26.64 ± 1.33</td>
</tr>
<tr>
<td>3</td>
<td>2500</td>
<td>0.222 ± 0.006</td>
<td>26.25 ± 2.50</td>
<td>28.53 ± 3.37</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0.172 ± 0.026</td>
<td>15.25 ± 1.49</td>
<td>23.01 ± 1.61</td>
</tr>
<tr>
<td>7</td>
<td>125</td>
<td>0.201 ± 0.006</td>
<td>18.12 ± 0.85</td>
<td>31.58 ± 1.22*</td>
</tr>
<tr>
<td>7</td>
<td>2500</td>
<td>0.318 ± 0.009*</td>
<td>33.74 ± 1.03*</td>
<td>62.56 ± 1.91*</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0.203 ± 0.016</td>
<td>15.91 ± 1.12</td>
<td>24.90 ± 1.67</td>
</tr>
<tr>
<td>10</td>
<td>125</td>
<td>0.276 ± 0.015*</td>
<td>21.83 ± 0.56*</td>
<td>37.74 ± 2.04*</td>
</tr>
<tr>
<td>10</td>
<td>2500</td>
<td>0.371 ± 0.009*</td>
<td>32.17 ± 1.34*</td>
<td>64.98 ± 3.96*</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>0.239 ± 0.005</td>
<td>20.13 ± 0.57</td>
<td>28.24 ± 1.13</td>
</tr>
<tr>
<td>21</td>
<td>125</td>
<td>0.295 ± 0.009*</td>
<td>23.10 ± 0.42*</td>
<td>40.40 ± 1.25*</td>
</tr>
<tr>
<td>21</td>
<td>2500</td>
<td>0.469 ± 0.012</td>
<td>38.40 ± 1.18*</td>
<td>90.38 ± 2.95*</td>
</tr>
</tbody>
</table>

\*° Significantly different from control at \*\((p < 0.05)\), \*\((p < 0.01)\), or \*\((p < 0.001)\).
carcinogenicity, and enzyme induction were examined in livers from B6C3F1 mice (NTP, 1993; Griffin and Burka, 1993; Cunningham et al., 1994; Griffin et al., 1995; Bucher et al., 1994). In each of these studies, increases in liver size relative to overall body weight were noted. This repeatable and consistent response was used (Table 1) to show that the animals consumed the dosed feed in equivalent amounts and indicates that the results of these studies can be directly compared.

The results of these earlier investigations indicate that the biochemical effects of repeated oxazepam treatment are similar to the effects of repeated exposure to phenobarbital. For example, both compounds exert their sedative effects by an action on the GABA receptor in the CNS (Harvey, 1985). Both are mouse liver carcinogens and induce hyperplasia of the liver and thyroid gland in mice (NTP, 1993; Bucher et al., 1994; Rice et al., 1994; McClain, 1989). Liver tumors induced by phenobarbital and oxazepam both have a decreased incidence of activated H-ras oncogenes (Devereux et al., 1994; Fox et al., 1990). Induction of drug metabolizing enzymes is another characteristic shared by these drugs. The overall spectrum of induction by oxazepam appears to greatly resemble that of the pleotropic response seen for a group of compounds typified by phenobarbital but including DDT, α-hexachlorocyclohexane, clotrimazole, and clonazepam (Jones and Lubet, 1992). The properties that oxaze-

### TABLE 3

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Dose (ppm)</th>
<th>nmol b5/mg homogenate</th>
<th>nmol b5/g liver</th>
<th>nmol b5/liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>0.053 ± 0.004</td>
<td>4.43 ± 0.75</td>
<td>6.62 ± 1.11</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>0.050 ± 0.005</td>
<td>3.95 ± 0.36</td>
<td>5.69 ± 0.35</td>
</tr>
<tr>
<td>3</td>
<td>2500</td>
<td>0.048 ± 0.008</td>
<td>5.54 ± 0.90</td>
<td>6.61 ± 0.98</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0.037 ± 0.007</td>
<td>3.41 ± 0.66</td>
<td>5.06 ± 0.78</td>
</tr>
<tr>
<td>7</td>
<td>125</td>
<td>0.049 ± 0.008</td>
<td>4.29 ± 0.44</td>
<td>7.49 ± 0.79</td>
</tr>
<tr>
<td>7</td>
<td>2500</td>
<td>0.103 ± 0.004</td>
<td>10.99 ± 0.40</td>
<td>20.38 ± 0.70</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0.061 ± 0.005</td>
<td>4.84 ± 0.44</td>
<td>7.56 ± 0.56</td>
</tr>
<tr>
<td>10</td>
<td>125</td>
<td>0.077 ± 0.003</td>
<td>6.15 ± 0.49</td>
<td>10.53 ± 0.55</td>
</tr>
<tr>
<td>10</td>
<td>2500</td>
<td>0.118 ± 0.009</td>
<td>10.23 ± 0.91</td>
<td>20.77 ± 2.35</td>
</tr>
<tr>
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<td>0</td>
<td>0.065 ± 0.006</td>
<td>5.50 ± 0.55</td>
<td>7.77 ± 0.95</td>
</tr>
<tr>
<td>21</td>
<td>125</td>
<td>0.058 ± 0.006</td>
<td>4.50 ± 0.49</td>
<td>7.89 ± 0.86</td>
</tr>
<tr>
<td>21</td>
<td>2500</td>
<td>0.123 ± 0.016</td>
<td>10.17 ± 1.57</td>
<td>23.85 ± 3.39</td>
</tr>
</tbody>
</table>

*" Significantly different from control at "(p < 0.05), "(p < 0.01), "(p < 0.001).

### TABLE 4

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Dose (ppm)</th>
<th>Lipid peroxidation*</th>
<th>Superoxide dismutase†</th>
<th>Catalase‡</th>
<th>Glutathione peroxidase§</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>24.2 ± 5.8</td>
<td>2.26 ± 0.10</td>
<td>72.5 ± 11.3</td>
<td>8.89 ± 2.16</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>34.2 ± 10.9</td>
<td>2.45 ± 0.36</td>
<td>52.2 ± 5.6</td>
<td>6.74 ± 1.03</td>
</tr>
<tr>
<td>3</td>
<td>2500</td>
<td>47.0 ± 13.9</td>
<td>2.62 ± 0.23</td>
<td>61.0 ± 5.1</td>
<td>4.25 ± 1.04</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>100.0 ± 20.8</td>
<td>2.55 ± 0.10</td>
<td>54.6 ± 6.4</td>
<td>8.20 ± 0.90</td>
</tr>
<tr>
<td>7</td>
<td>125</td>
<td>102.0 ± 8.1</td>
<td>2.68 ± 0.20</td>
<td>70.1 ± 8.5</td>
<td>13.90 ± 1.44</td>
</tr>
<tr>
<td>7</td>
<td>2500</td>
<td>100.0 ± 29.0</td>
<td>2.68 ± 0.10</td>
<td>91.2 ± 7.6</td>
<td>6.59 ± 1.26</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>58.5 ± 11.1</td>
<td>1.90 ± 0.26</td>
<td>61.1 ± 4.1</td>
<td>6.42 ± 0.94</td>
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<tr>
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<td>125</td>
<td>47.5 ± 6.2</td>
<td>1.96 ± 0.10</td>
<td>66.2 ± 2.5</td>
<td>6.40 ± 0.74</td>
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<tr>
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<td>2500</td>
<td>40.6 ± 14.8</td>
<td>1.93 ± 0.03</td>
<td>101.2 ± 5.8</td>
<td>5.33 ± 0.91</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>11.5 ± 17.3</td>
<td>2.48 ± 0.10</td>
<td>68.8 ± 6.4</td>
<td>5.32 ± 0.92</td>
</tr>
<tr>
<td>21</td>
<td>125</td>
<td>7.6 ± 5.6</td>
<td>2.81 ± 0.16</td>
<td>72.7 ± 6.0</td>
<td>4.27 ± 0.16</td>
</tr>
<tr>
<td>21</td>
<td>2500</td>
<td>9.4 ± 1.4</td>
<td>2.78 ± 0.03</td>
<td>89.2 ± 7.8</td>
<td>5.79 ± 1.04</td>
</tr>
</tbody>
</table>

* Nanomoles of TBA-reactive products/gram of liver.
† Nanomoles of adrenochrome formed/minute.
‡ Micromoles of H2O2 reduced/mg of homogenate/minute.
§ Micromoles of glutathione oxidized/milligram of homogenate/minute.
**" Significantly different from control at "(p < 0.05), "(p < 0.01), or "(p < 0.001).
Proliferation, Enzyme Induction, and TSH in Oxazepam-Treated Mice

Oxazepam shares with these compounds include induction of total P450, CYP2B, glutathione transferase, glucuronyl transferase, and increased liver-to-body weight ratios (Griffin et al., 1995). Although there is some variation in the response, a single mechanism for induction of the pleotropic response at the molecular level has been proposed (He and Fulco, 1991; Upadhya et al., 1992).

In our previous study of oxazepam induction there was, however, an inconsistency. Specific activities of cytochrome P450, cytochrome b5, aminopyrene demethylase, aniline hydroxylase, glucuronyl transferase, and cytosolic glutathione transferase were shown to be transiently induced by oxazepam-dosed feed. By 21 days of treatment, all of the activities returned to control or less than control levels (Griffin et al., 1995). Transient induction by oxazepam is inconsistent with both the effects of phenobarbital and the in vivo evidence of sustained enzyme induction by oxazepam (Cunningham et al., 1994; Diwan et al., 1986). The possibility that sustained enzyme induction was the result of increased microsomal protein per gram of liver and increased liver size rather than due to increased specific activities of enzymes was addressed in this study. In the current study, microsomal, but not cytosolic protein contents were elevated by dosed-feed treatment (Table 1). The increases in microsomal protein content along with increases in liver size meant that the activities of the microsomal enzymes were still increasing when expressed as activity per gram of liver or as activity per liver. In Tables 2 and 3 this is demonstrated for two representative microsomal enzymes, cytochromes P450 and b5. At 21 days there are up to 3x increases in activity per liver even though specific activities as previously reported were at control or less than control (Griffin et al., 1995). Cytosolic protein content did not change; therefore, the specific activity of cytosolic glutathione transferase is a good indicator of total activity as long as increases in liver size are taken into account. These results indicate that the transient induction of microsomal enzymes detected previously was an artifact of the units of activity used (activity/mg of microsomal protein rather than activity/g of liver). Enzyme induction by oxazepam in mice is sustained during chronic exposure in the same manner as that by phenobarbital.

One possible mechanism of oxazepam-mediated carcinogenicity was examined as a result of these many similarities between phenobarbital and oxazepam. Phenobarbital is known to induce hepatic metabolism and excretion of thyroxine, resulting in compensatory increased TSH secretion by the pituitary which leads in turn to thyroid tumors (McClain, 1989; McClain et al., 1989). This increase in thyroid hormone metabolism resulted primarily from induction of UDP-glucuronyltransferases in the liver. These enzymes, responsible for the conjugation and elimination of thyroxine, were also induced during exposure to oxazepam in mice based on increased elimination of glucuronidated oxazepam metabolites after 14 days of treatment (Griffin and Burka, 1993). In this study, p-nitrophenol glucuronyl transferase activity was elevated more than twofold by 10 days of treatment with oxazepam. This particular assay measures activity of the glucuronyl transferase isozyme that is responsible for glucuronidation of thyroxine (Van Raaij et al., 1993). Decreased circulation of thyroxine, secondary to increased glucuronidation of thyroxine, is believed to be responsible for stimulating the pituitary to release TSH into the blood of rats (McClain et al., 1989). These effects were also observed in the present study (Fig. 2 and Results). Even-

![Fig. 1. Hepatic nonprotein sulfhydryl levels following exposure to oxazepam-dosed feed, n = 5.](image-url)

![Fig. 2. Hepatocyte labeling index and serum TSH in male B6C3F1 mice treated with 2500 ppm oxazepam in the diet for 3, 7, 10, and 21 days. *p < 0.02 compared to age-matched untreated controls; n = 5. **p < 0.01 compared to age-matched untreated controls; n = 5.](image-url)
tually, thyroid hormone homeostasis was reestablished and plasma TSH levels also returned to control by Day 21 in the oxazepam-treated animals. These responses in oxazepam-treated mice in many respects appeared to be the same as the phenobarbital-mediated responses in which induced UDP-glucuronyltransferase resulted in increased thyroxine–glucuronide excretion in rats (McClain, 1989). This suggests that the thyroid hyperplasia and thyroid tumors in oxazepam treated mice occurred via the same mechanism as that reported in phenobarbital treated rats.

The temporal sequence of plasma TSH levels is mirrored in the hepatic cell proliferation events. Plasma TSH is elevated by 10 days and thereafter returns to control by 21 days (Fig. 2). Simultaneously, hepatic cell proliferation is significantly elevated by 10 days of oxazepam treatment, is decreasing but still elevated compared to control by 21 days (Fig. 2), and returns to control levels by 28 days (Cunningham et al., 1994). Liver-to-body weight ratios, however, remained elevated out to 90 days and reflected an increase in cell size as well as cell number. Phenobarbital treatment produces a similar time course of hepatic cell proliferation (Smith et al., 1991) and elevates TSH (McClain, 1989).

It is possible, though speculative, that TSH is exerting a trophic effect on hepatocytes. The combination of a benzodiazepine (oxazepam) or a barbiturate (phenobarbital) with a trophic hormone (TSH) may be necessary for hepatic mitogenesis. However, although this requirement for a synergistic effect has been observed for prolactin and peripheral benzodiazepine receptor activation in vitro, it has not been documented in vivo (Laird et al., 1989). The lack of an effect of the noncarcinogenic dose (125 ppm) on TSH levels further supports this hypothesis. Although a role for TSH in oxazepam hepatocarcinogenesis is highly speculative, this potential mechanism of hepatocarcinogenicity may warrant further investigation.

The role of thyroid hormones in the induction of these rodent liver and thyroid tumors may be especially important for human risk assessment. It has been proposed that rodent tumors due to thyroid disturbances are of no relevance to humans because in man thyroid binding globulin acts as a buffer to maintain plasma thyroid hormone levels (Swenberg et al., 1992). This protein is not present in rodents rendering them much more susceptible to thyroid hormone imbalances.

There is little evidence to indicate that oxazepam acts through a genotoxic mechanism. However, evidence of chronic enzyme induction by oxazepam raised concern that oxazepam treatment might yield reactive metabolites or free radicals only after oxazepam-mediated induction. In an effort to determine if free radicals play a role in oxazepam-mediated tumorigenesis after dosed-feed exposure we measured radicals only after oxazepam-mediated induction. In an effort oxazepam treatment might yield reactive metabolites or free radicals. However, evidence of an association or linkage between catalase and one of the other induced enzymes, rather than the presence of free radicals. Overall, there was little evidence of free radical involvement in the carcinogenicity of oxazepam in mice. Another possible contributing factor to the carcinogenicity of oxazepam is depletion of glutathione by interaction with reactive metabolites resulting in an increased susceptibility to normal levels of oxidative stress. The effects of oxazepam-dosed feed treatment on NPS levels are presented in Fig. 1. A consistent decrease in the NPS content of the livers was detected at the high dose. However, the magnitude of the decrease was relatively small (<20%) in each case, except at 3 days. Here the larger decrease in NPS (approximately 40%) was more likely the result of the initial decrease in food consumption due to taste aversion and/or sedation. Apart from the decrease in the first 3 days, the depletion of glutathione at the high dose was probably not of sufficient magnitude to significantly reduce the ability of hepatocytes to resist oxidative stress. Overall, there was little or no evidence of increased oxidative stress after induction by oxazepam.

Although the oxidative stress studies were negative, they do provide another opportunity to compare oxazepam and phenobarbital. In a phenobarbital-dosed feed study (0.05% in water for 10 weeks), lipid peroxidation products were statistically decreased in all three strains of mice examined (Ahotupa et al., 1993). The decreases were quantitatively similar to the not statistically significant decreases at 10 and 21 days in this study (Table 4). Superoxide dismutase was not assayed in the study by Ahotupa et al. (1993) but glutathione peroxidase activity was unchanged. Catalase activity was significantly elevated in all three strains of mice just as in this study following oxazepam treatment. Reduced glutathione was not significantly altered in two of the three strains following oxazepam treatment. In contrast, chronic treatment of rats with phenobarbital elevated most indicators of oxidative stress (Furakawa et al., 1985; Junqueira et al., 1991). Given minor differences due to different techniques and interlaboratory variability the effects of oxazepam on indicators of oxidative stress in mice are very similar to the effects of phenobarbital on these same indicators. The very similar
elevations in catalase activities in the absence of elevated oxidative stress are especially significant. Induction of catalase may be another, as yet unidentified aspect of the pleotropic response.

The mechanism of carcinogenesis of “noncytotoxic, nongenotoxic” carcinogens has been the subject of much research interest. The hypothesis that noncytotoxic, nongenotoxic carcinogens stimulate clonal expansion of spontaneous preneoplastic cells has been widely proposed, but recent data from two laboratories have undermined this theory. Devereux et al. (1994) demonstrated that tumors in B6C3F1 mice induced by oxazepam had fewer mutations in codon 61 of the H-ras oncogene compared to tumors from control mice. Additional evidence demonstrated the ras oncogene was hypomethylated in B6C3F1 mice following partial hepatectomy or administration of phenobarbital (Ray et al., 1994), indicating that DNA hypomethylation may be a critical mechanism of carcinogenicity by noncytotoxic, nongenotoxic carcinogens like phenobarbital and oxazepam. Additional studies are now in progress to address this issue.

In summary, this study characterized several biochemical events following the initiation of oxazepam-dosed feed treatment in mice. Total activity of microsomal enzyme systems was induced over the 21-day period in which they were examined primarily due to increased protein content. The pattern of induction appears to be typical of that seen for phenobarbital-type inducers. There was little evidence of increased oxidative stress over this time period. Cell proliferation at the high dose was maximal at 10 days of treatment. Plasma TSH levels were also elevated at this dose after 10 days. These data, along with previously described work, suggest that in mice chronic exposure to oxazepam induces a similar spectrum of biochemical effects including the same drug-metabolizing enzymes as exposure to phenobarbital (Griffin et al., 1995). These results suggest that oxazepam- and phenobarbital-mediated hepatocarcinogenesis may occur via the same as yet uncharacterized mechanism. Disturbances in the thyroid–pituitary endocrine axis may also directly relate to the thyroid gland hyperplasia/carcinogenesis induced by these two chemicals in mice.

Phenobarbital is not a human liver carcinogen (Clemmesen and Hjalgrim-Jensen, 1978, 1980; Olsen et al., 1993). Therefore, if oxazepam-mediated carcinogenesis in mice occurs by the same mechanism as carcinogenesis mediated by phenobarbital as this study suggests may be a possibility, then oxazepam is not likely to be a human carcinogen. Therefore, these results may be relevant for the human safety assessment of oxazepam.

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REFERENCES


