DOSE-DEPENDENT INDUCTION OF CARCINOMAS AND GLUTATHIONE S-TRANSFERASE PLACENTAL FORM NEGATIVE EOSINOPHILIC FOCI IN THE RAT LIVER BY DI(2-ETHYLHEXYL)PHTHALATE AFTER DIETHYLNITROSAMINE INITIATION

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ABSTRACT — The dose-dependence of di(2-ethylhexyl)phthalate (DEHP) hepatocarcinogenicity was investigated in male F344 rats which were initially injected with diethylnitrosamine (200 mg/kg, ip) and subjected to partial hepatectomy at week 3. The animals were administered DEHP in the diet at concentrations of 30, 300, 3000, or 12000 ppm starting 2 weeks after the DEN injection for up to 46 weeks and killed at weeks 8, 24, 48 and 52. Additional groups were given clofibrate (3000 ppm in diet) or basal diet instead of the DEHP diet. Incidences of hepatocellular carcinomas were 75% (9/12, P<0.01) for 12000 ppm, 10% (1/10) for 3000 ppm, 7% (1/14) for 300 ppm, 0% (0/13) for 30 ppm, 15% (2/13) for clofibrate, and 8% (1/13) for the basal diet group at week 52, 4 weeks after cessation of chemical feeding. Development of glutathione S-transferase placental form (GST-P) positive foci was only slightly increased by clofibrate-administration at week 52 and consistently lower than the control level in the DEHP-treated groups after 24 weeks. In contrast, GST-P negative eosinophilic foci were dose-dependently increased in the more than 300 ppm DEHP and clofibrate treated groups. At the 30 ppm dose level, however, no morphological changes were apparent in the liver. Thus, the non-observed effect level regarding the promotional activity of hepatocarcinogenesis was demonstrated at 30 ppm, the effects being predictable on the basis of development of GST-P negative eosinophilic foci.

KEY WORDS: DEHP, Dose-response study, Hepatocarcinogenesis, Altered hepatic foci, GST-P positive and negative foci, Peroxisome proliferator, Rat

INTRODUCTION

Di (2-ethylhexyl)phthalate (DEHP) is the most widely used plasticizer for polyvinylchloride. When administered to experimental animals, it induces marked hepatocellular hypertrophy and hyperplasia in the liver, hepatic peroxisome proliferation and an increase in the activities of some peroxisome-associated enzymes (Ashby et al., 1994; Bentley et al., 1993; Moody et al., 1991; Rao and Reddy, 1987). Hepatocarcinogenicity of DEHP has been demonstrated in both sexes of B6C3F1 mice and F344 rats fed 3000 ppm and 6000 ppm, respectively, for more than 100 weeks (Kluwe et al., 1982) and in male Sprague-Dawley rats receiving 2% in the diet for 2 years (Lake et al., 1987). However, DEHP was not found to be carcinogenic in Syrian golden hamsters with i.p. (maximum total dose 54 g/kg) or inhalation (7-10 mg/kg) routes of application (Schmerzer et al., 1988). DEHP and other peroxisome proliferators are not mutagenic in either in vitro or in vivo studies nor do they bind to liver DNA in vivo (Ashby et al., 1994; Bentley et al., 1987).
1993; Butterworth et al., 1984; Cattley and Preston, 1995; Moody et al., 1991; Rao et al., 1987; Schmerzer et al., 1988). They are therefore considered to be typical nongenotoxic carcinogens.

While there is evidence to suggest that their carcinogenicity may be caused by intracellular production of oxygen radicals from hydrogen peroxide generated by the increased numbers of peroxisomes (Ashby et al., 1994; Bentley et al., 1993; Cattley et al., 1995; Kasai et al., 1989; Lake et al., 1987; Moody et al., 1991; Rao et al., 1987; Takagi et al., 1990), the mechanisms by which DEHP causes liver neoplasms in rodents are still unknown.

It has been pointed out that DEHP may rather inhibit the development of γ-glutamyltranspeptidase-positive or glutathione S-transferase placental form (GST-P)-positive liver cell foci and nodules in rats when given alone (Rao et al., 1986; Rao et al., 1987) or subsequent to initiating carcinogens such as diethylnitrosamine (DEN) (Deangelo and Garrett, 1983; Gerbracht et al., 1990; Oesterle and Deml, 1988; Popp et al., 1985) or 2-acetylaminofluorene (Williams et al., 1987). Similar properties were demonstrated for other peroxisome proliferators alone (Greaves et al., 1986; Rao et al., 1986) or following DEN (Cattley et al., 1994; Glaubet et al., 1986), 2-acetylaminofluorene (Cattley et al., 1994), aflatoxin B1 (Grasl-Kraupp et al., 1990) or N-nitrosomorpholine (Gerbracht et al., 1990) initiation. We have demonstrated that GST-P expression, an excellent marker for preneoplastic lesions in the rat liver (Hasegawa and Ito, 1992; Ito et al., 1988; Shirai, 1997; Tatematsu et al., 1985), cannot be applied for prediction of hepatocarcinogenicity of peroxisome proliferators (Hasegawa et al., 1994; Hosokawa et al., 1989; Ito et al., 1992; Shirai, 1997). Alternatively, some types of foci, such as adenosine triphosphatase deficient, glucose-6-phosphate dehydrogenase positive, glucose-6-phosphatase deficient, or basophilic hepatic lesions, have been proposed as superior indicators for hepatocarcinogenicity of peroxisome proliferators (Cattley et al., 1994; Gerbracht et al., 1990; Glaubet et al., 1986; Grasl-Kraupp et al., 1990; Greaves et al., 1986; Marsman et al., 1988).

In the present experiment, a precise analysis of altered hepatic foci based on histopathological appearance and GST-P positivity was performed for rats given DEHP at 4 dose levels in the diet after DEN initiation. Animals were killed sequentially over a period of 52 weeks to establish a non-observed effects level for this chemical and to identify an appropriate simply-detectable marker lesion for prediction of peroxisome proliferator hepatocarcinogenicity.

**MATERIALS AND METHODS**

**Chemicals**

DEHP, clofibrate and DEN were obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. The lot numbers of DEHP and clofibrate were FGCO1 and FCX01, respectively.

**Two-stage carcinogenicity study**

A total of 270 male F344/DuCrlj rats, aged 5 weeks, were obtained from Charles River Japan Inc., Atsugi, Japan, and housed 5 per cage with wood-chip bedding in an air-conditioned animal room at 22±2°C and 55±10% humidity with 12hr/12hr light-dark cycle. When the rats were 6 weeks old, the experiment was started. The experimental protocol, shown in Fig. 1, is based on our rapid bioassay system for detection of hepatocarcinogens (Hasegawa and Ito, 1992; Ito et al., 1997; Ito et al., 1988; Shirai, 1997). All rats were initially intraperitoneally administered DEN at a dose of 200 mg/kg body weight and subjected to 2/3 partial hepatectomy at week 3. Chemical feeding was started 2 weeks after the DEN injection. Forty-five rats per dose group were administered DEHP mixed in the diet (Oriental MF powdered diet, Oriental Yeast Co., Tokyo, Japan) at concentrations of 12000, 3000, 300 or 30 ppm while the control group rats were fed on basal powdered diet. Another 45 rats were given clofibrate, 3000 ppm in the diet, as a positive control. The chemical feeding was continued until week 48 when the rats were returned to the basal diet until week 52. Food and water were available ad libitum throughout the experiment. Body weights and food consumption were recorded every week during the first 14 weeks and thereafter.

![Experimental protocol](image-url)
every 2 weeks until the end of the experiment.

Subgroups of rats were killed at the ends of weeks 8, 24, 48 and 52 after overnight fasting. At sacrifice, the animals were macroscopically examined and all pathological lesions were removed for histopathological examination. Body and liver weight were determined. Three slices of liver were fixed in ice-cold acetone for immunohistochemical demonstration of GST-P. The remainders of the livers were fixed in 10% phosphate buffered formalin for histopathological examination of serially sectioned slides stained with hematoxylin and eosin (H&E) or for GST-P immunohistochemically. Sections were taken from three standard liver slices as well as demonstrating regions. In the standard sections, altered hepatic foci were classified into eosinophilic, basophilic and clear cell categories according to published criteria (Bannach, 1986; Harada et al., 1989). Mixed eosinophilic and clear cell type lesions were counted as eosinophilic foci (Hasegawa et al., 1994). Histopathological diagnosis of neoplastic lesions was made using all H&E sections including these with macroscopic changes, according to published criteria (Goodman et al., 1994). The total areas of liver sections assessed were measured with the aid of a video image processor (SPPICA-II, Nippon Avionics Co., Ltd., Tokyo), to allow numbers of foci per unit area to be calculated.

**GST-P immunohistochemistry**

GST-P immunohistochemistry was performed for acetone and formalin fixed slides as previously described (Tatematsu et al., 1985). For acetone fixed sections, GST-P positive foci more than 0.2 mm in diameter and the total areas of the liver sections examined were quantitated using a video image processor, and the data expressed as numbers and areas (mm²) per unit area of liver (cm²). The formalin fixed sections were used for identification of altered hepatic foci in serial sections stained with H&E and for GST-P.

**Statistical analysis**

The significance of differences between control and treated groups for each parameter was analyzed, and evaluated at P<0.05 or P<0.01. Analysis of variance for mean values of each group was performed using the F-test (Yoshimura, 1987). In a case of equal variance the Student's t-test was applied, and if not the Welch-test was used (Yoshimura, 1987). For incidence data, Fisher's exact probability test was employed (Yoshimura, 1987).

**RESULTS**

The highest doses of DEHP (12000 ppm) and clofibrate were toxic and the body weight gain of the treated animals was retarded throughout the experiment. At week 48 when the chemical feeding was stopped, body weights were significantly reduced at 350.2±33.4 g (P<0.01) for the DEHP (12000 ppm), and 392.4±12.9 g (P<0.01) for the clofibrate group as compared to the 425.8±25.0 g of the controls. The body weights demonstrated slight recovery after cessation of the peroxisome proliferator feeding. Dose-dependent increase of liver weights was observed for DEHP at week 48, comparable values being obtained for DEHP at 300 ppm and clofibrate (3000 ppm) (Fig. 2), but had decreased to the control level 4 weeks after cessation of chemical feeding. Food consumption did not significantly differ among the groups. Average chemical intakes (mg/kg/day) were 617.7 for 12000
ppm DEHP, 130.8 for 3000 ppm DEHP, 12.9 for 300 ppm DEHP, 1.3 for 30 ppm DEHP and 140.8 for clofibrate (3000 ppm).

On histopathological examination, eosinophilia and hypertrophy of hepatocytes were observed in the groups receiving 300 ppm DEHP or more and in the clofibrate-treated animals during the period of test chemical exposure. The background livers were histopathologically normal after cessation of treatment.

Incidences of neoplastic lesions found in the liver are summarized in Table 1. At week 24, neoplastic nodules were observed in all groups, including the DEN controls. At week 48, hepatocellular carcinomas were induced in the DEHP (12000 ppm) and clofibrate treated groups, and a clear DEHP dose-dependence was observed for the neoplastic nodule incidence. By week 52, hepatocellular carcinomas had developed in 9 of 12 rats (75%) in the 12000 ppm DEHP group (P<0.01), 2 of 13 rats (15%) in the clofibrate group, and 8% (1/13) of the controls.

Quantitative data for GST-P positive foci in the acetone-fixed sections are summarized in Fig. 3. At week 8, there were no significant changes in the DEHP treatment groups, whereas a slight decrease was evident with the clofibrate treatment. At week 24, both the numbers and areas of GST-P positive foci were dose-dependently decreased and significantly lower than the control values in the DEHP at 3000 and 12000 ppm dose groups. At week 48, the decrease in number of GST-P positive foci was much clearer for DEHP, although

![Fig. 3. Sequential changes in numbers (A) and areas (b) of GST-P positive foci in the livers of rats treated with DEHP or clofibrate after DEN initiation. Significantly different from the control group at P<0.05(*) and P<0.01(**).](image)

<table>
<thead>
<tr>
<th>Week</th>
<th>Lesions</th>
<th>Control</th>
<th>DEHP</th>
<th>Clofibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 ppm</td>
<td>300 ppm</td>
<td>3000 ppm</td>
</tr>
<tr>
<td>24</td>
<td>No. of rats</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>3 (25)</td>
<td>2 (17)</td>
<td>3 (25)</td>
</tr>
<tr>
<td></td>
<td>HCC</td>
<td>1 (20)</td>
<td>2 (40)</td>
<td>3 (60)</td>
</tr>
<tr>
<td></td>
<td>HA/HCC</td>
<td>1 (20)</td>
<td>2 (40)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>48</td>
<td>No. of rats</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>1 (20)</td>
<td>2 (40)</td>
<td>3 (60)</td>
</tr>
<tr>
<td></td>
<td>HCC</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HA/HCC</td>
<td>1 (20)</td>
<td>2 (40)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>52</td>
<td>No. of rats</td>
<td>13</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>6 (46)</td>
<td>8 (62)</td>
<td>10 (71)</td>
</tr>
<tr>
<td></td>
<td>HCC</td>
<td>1 (8)</td>
<td>0</td>
<td>1 (7)</td>
</tr>
<tr>
<td></td>
<td>HA/HCC</td>
<td>7 (54)</td>
<td>8 (62)</td>
<td>10 (71)</td>
</tr>
</tbody>
</table>

HA; Hepatocellular adenoma, HCC; Hepatocellular carcinoma.
Significantly different from the control group at \(a\); P<0.05, \(b\); P<0.01.
Dose-response study of DEHP Hepatocarcinogenicity.

no treatment-related changes were found for clofibrate. At week 52, numbers of GST-P positive foci were still lower in the DEHP-treated groups than in the controls, but were significantly increased in the clofibrate-treated group.

Results for GST-P positive and negative hepatic foci counted in the formalin-fixed sections are shown in Fig. 4. Even in the DEN control group, numbers of positive foci continuously increased with time, although negative foci were most frequent at week 48. The induction of GST-P positive and negative foci was slightly enhanced by DEHP and clofibrate administration after 48 weeks.

Details for altered hepatocyte foci are summarized in Table 2. At week 8, numbers of eosinophilic foci (larger than 10 cells) were dose-dependently decreased in the DEHP-treated groups, although values for GST-P negative eosinophilic foci were significantly increased. Similar results were obtained for clofibrate. No significant changes were observed for basophilic foci which were all GST-P negative throughout the experiment. There were GST-P positive foci for which no lesions were apparent in the serial sections stained with H&E.

At week 24, a similar tendency to that at week 8 was found. In particular, an increase in number of GST-P negative eosinophilic foci due to DEHP treatment was significant in the 12000 and 3000 ppm groups, while the 300 and 30 ppm doses were without effect. Numbers of basophilic foci were slightly increased in all groups.

At week 48, increase in GST-P negative eosinophilic foci was again evident with all doses but 30 ppm.

At week 52, both eosinophilic and basophilic foci were clearly increased in number, even in the controls, as compared to the week 48 values. Numbers of GST-P negative eosinophilic foci were again elevated in all groups but that receiving 30 ppm.

DISCUSSION

The NTP carcinogenicity study of DEHP in F344 rats resulted in hepatocellular carcinoma incidences of 1/50, 1/49 and 5/49 in males and 0/50, 2/49 and 8/50 in females, respectively in control, low (6000 ppm)- and high (12000 ppm)- dose groups after 103 weeks (Kluwe et al., 1982). In the present two-stage protocol with DEN initiation and 4 doses of DEHP, an apparent enhancing effect on liver carcinogenicity was similarly
### Table 2. Positivity for GST-P in the altered hepatocellular lesions

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>No. of rats examined</th>
<th>GST-P positivity</th>
<th>Number of foci in formalin-fixed livers (Nos/cm²)</th>
<th>Not detectable on H&amp;E</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Control</td>
<td>5</td>
<td>+</td>
<td>6.97 ± 0.07</td>
<td>0.87 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>DEHP 30 ppm</td>
<td>5</td>
<td>+</td>
<td>7.57 ± 0.25</td>
<td>0.70 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>DEHP 300 ppm</td>
<td>5</td>
<td>+</td>
<td>5.82 ± 0.23</td>
<td>0.22 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>DEHP 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>4.30 ± 1.34a</td>
<td>0.32 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>DEHP 12000 ppm</td>
<td>5</td>
<td>+</td>
<td>3.87 ± 1.16a</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td>Clofibrate 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>3.10 ± 0.94b</td>
<td>0a</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>5</td>
<td>+</td>
<td>9.84 ± 3.11</td>
<td>2.33 ± 1.25</td>
</tr>
<tr>
<td></td>
<td>DEHP 30 ppm</td>
<td>5</td>
<td>+</td>
<td>11.56 ± 3.43</td>
<td>0.83 ± 0.65a</td>
</tr>
<tr>
<td></td>
<td>DEHP 300 ppm</td>
<td>5</td>
<td>+</td>
<td>11.66 ± 3.34</td>
<td>1.31 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>DEHP 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>9.06 ± 2.77</td>
<td>0.51 ± 0.73a</td>
</tr>
<tr>
<td></td>
<td>DEHP 12000 ppm</td>
<td>5</td>
<td>+</td>
<td>8.03 ± 3.16</td>
<td>0.46 ± 0.14a</td>
</tr>
<tr>
<td></td>
<td>Clofibrate 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>11.94 ± 3.64</td>
<td>0.58 ± 0.56a</td>
</tr>
<tr>
<td>48</td>
<td>Control</td>
<td>4</td>
<td>+</td>
<td>19.74 ± 6.23</td>
<td>1.27 ± 1.12</td>
</tr>
<tr>
<td></td>
<td>DEHP 30 ppm</td>
<td>5</td>
<td>+</td>
<td>24.76 ± 7.20</td>
<td>0.45 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>DEHP 300 ppm</td>
<td>5</td>
<td>+</td>
<td>17.84 ± 5.52</td>
<td>1.05 ± 1.23</td>
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<tr>
<td></td>
<td>DEHP 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>22.34 ± 11.41</td>
<td>0.54 ± 0.35</td>
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<tr>
<td></td>
<td>DEHP 12000 ppm</td>
<td>5</td>
<td>+</td>
<td>13.83 ± 4.94</td>
<td>0.12 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>Clofibrate 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>21.46 ± 5.11</td>
<td>0.09 ± 0.19</td>
</tr>
<tr>
<td>52</td>
<td>Control</td>
<td>9</td>
<td>+</td>
<td>22.21 ± 7.38</td>
<td>2.89 ± 2.04</td>
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<tr>
<td></td>
<td>DEHP 30 ppm</td>
<td>5</td>
<td>+</td>
<td>21.00 ± 6.19</td>
<td>0.51 ± 0.63b</td>
</tr>
<tr>
<td></td>
<td>DEHP 300 ppm</td>
<td>5</td>
<td>+</td>
<td>21.22 ± 5.23</td>
<td>0.94 ± 1.07</td>
</tr>
<tr>
<td></td>
<td>DEHP 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>26.73 ± 6.41</td>
<td>1.43 ± 2.02</td>
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<tr>
<td></td>
<td>DEHP 12000 ppm</td>
<td>10</td>
<td>+</td>
<td>20.65 ± 5.21</td>
<td>0.81 ± 0.63a</td>
</tr>
<tr>
<td></td>
<td>Clofibrate 3000 ppm</td>
<td>5</td>
<td>+</td>
<td>32.34 ± 6.68a</td>
<td>1.96 ± 2.03</td>
</tr>
</tbody>
</table>

Significantly different from the control group at a, P<0.05 and b, P<0.01.
Dose-response study of DEHP Hepatocarcinogenicity.

only observed at a concentration of 12000 ppm after a 52 week observation period. Significant increase of liver weights and the histopathological changes, hepatocellular hypertrophy and eosinophilia, were observed in the 300 ppm and above doses and in animals receiving clofibrate treatment. At the 30 ppm dose level, no significant influence was detected on any of the parameters in the present experiment. Thus, the DEHP-caused carcinogenicity and/or promotional activity appeared to be related to effects on peroxisome proliferation.

GST-P has been shown to be a most reliable marker for detection of rat hepatic preneoplastic and neoplastic lesions (Ito et al., 1997; Shirai, 1997; Tatamatsu et al., 1985), and a rapid bioassay system of 8 weeks duration (medium-term liver bioassay system) has been established based on quantitative analysis of GST-P positive lesions as endpoint indicators (Ito et al., 1997; Ito et al., 1988; Shirai, 1997; Tatamatsu et al., 1985). As in the case of γ-glutamyltranspeptidase as the marker enzyme, however, the hepatocarcinogenicity of peroxisome proliferators has not been detectable based on GST-P positivity of foci (Rao et al., 1986). It appears that only a decrease in connexin expression is a common finding in both peroxisome proliferator and genotoxic carcinogen induced liver foci (Tsuda et al., 1996).

Although DEHP-treatment did not increase GST-P positive foci development, in line with previous observations (Hosokawa et al., 1989; Ito et al., 1992; Rao et al., 1986), its liver carcinogenicity could be predicted by increased induction of GST-P negative eosinophilic foci at doses of 3000 ppm and above. Previously, we demonstrated that DEN-induced GST-P positive foci were largely replaced by negative ones on subsequent treatment with clofibrate (3000 ppm in the diet) for more than 32 weeks, with the total number of foci being significantly increased (Hosokawa et al., 1989). In the present experiment with DEHP, however, induction of altered hepatocyte lesions itself was not increased by the time point of 52 weeks even at the highest dietary dose. Although some authors have proposed that the basophilic focus is a more reliable indicator of peroxisome proliferator hepatocarcinogenesis than the eosinophilic focus (Chen et al., 1994; Gerbracht et al., 1990; Grasl-Kraupp et al., 1990; Grasl-Kraupp et al., 1993; Greaves et al., 1986), this was not supported by our present observations. Our previous data (Hasegawa et al., 1994) demonstrated that eosinophilic foci are more reliable markers for carcinogenicity than basophilic foci based on the following findings: i) good correlation between GST-P positive foci induction and hepatocarcinogenicity for a number of chemicals was apparent; ii) basophilic foci were usually negative and eosinophilic foci were positive for GST-P immunohistochemistry and GGT histochemistry; and iii) basophilic foci were increased in number even in non-treated aged animals in this experiment. It has been shown that more than 90% of the liver foci induced by genotoxic liver carcinogens are positive for GST-P. However, the present data demonstrated only 44–68% of DEHP or clofibrate induced liver lesions to be positive. Together with the previous results, the present findings clearly indicate that GST-P is not a suitable marker for detection of liver lesions induced by chemicals that induce peroxisome proliferation.

It has been well described that phenotypic expression of enzymes within altered hepatic foci changes from positive to negative during peroxisome proliferator treatment (Gerbracht et al., 1990; Hosokawa et al., 1997). Chen et al. (Chen et al., 1994) reported that treatment with the peroxisome proliferator ciprofibrate resulted in two apparently opposing effects: inhibition of cell division in most GST-P positive nodules and promotion of cell division in certain other small GST-P negative lesions. These observations are very consistent with our present findings. The negativity is stable as observed by Yeldandi et al. (Yeldandi et al., 1989).

Overall increase in cell proliferation in the liver (Miller et al., 1996) or selective enhancement of cell division within altered foci (Grasl-Kraupp et al., 1993; Miller et al., 1996) have been considered to be very important factors in peroxisome proliferator hepatocarcinogenesis, as a part of a generally proposed hypothesis (Chen et al., 1994). Cellular injury caused by oxygen radicals generated by peroxisomes has been considered to be related to increased cell proliferation (Ashby et al., 1994; Bentley et al., 1993).

Bentley et al. (Bentley et al., 1993) proposed a hypothetical dose response curve for effects of peroxisome proliferators on the liver. At very low doses, administration of peroxisome proliferators will not lead to any changes. With increasing dose, however, stimulation of peroxisome proliferation and DNA synthesis occur. Only at the highest dose levels will liver tumors develop within the life span. Thus, with regard to biological effects, three dose levels were proposed, i.e. no effect level, peroxisome proliferation level and tumor induction level. The present findings of no apparent effects with 30 ppm DEHP and liver tumor developments with 12000 ppm are consistent with this hypothesis.

The effects of DEHP have been examined in many experiments, but usually doses of more than 2000 ppm
were applied (Deangelo et al., 1983; Gerbracht et al., 1990; Kluwe et al., 1982; Lake et al., 1987; Oesterle and Duml, 1988; Popp et al., 1985; Rao et al., 1987).

Humans have been found to be insensitive or unresponsive to peroxisome proliferators at the levels of exposure normal in daily life (Ashby et al., 1994; Bentley et al., 1993). Therefore, based on the present dose response findings and other reported data, it may be concluded that DEHP in the environment does not present as a hepatocarcinogenic hazard to man at the encountered doses.

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