Comparison of the Hepatotoxicity of Coumarin in the Rat, Mouse, and Syrian Hamster: A Dose and Time Response Study

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Comparison of the Hepatotoxicity of Coumarin in the Rat, Mouse, and Syrian Hamster: A Dose and Time Response Study.


The effects of coumarin treatment have been compared in male Sprague-Dawley CD rats, male CD-1 mice, and male Syrian hamsters. Rats were fed 0–0.75% coumarin for 1 and 4 weeks and 0–0.5% coumarin for 13 weeks, whereas mice and Syrian hamsters were fed 0–0.5 and 0–1.0% coumarin, respectively, for periods of 1, 4, and 13 weeks. In the rat, coumarin produced dose-related hepatotoxic effects which included vacuolar degeneration, apoptosis, and bile duct proliferation. These effects were particularly marked at dose levels of 0.3 and 0.5%, where liver tumors have been observed in a chronic study. Coumarin administration to rats also increased serum bilirubin content and both serum and hepatic γ-glutamyltransferase activity. While levels of hepatic total glutathione were increased by coumarin administration, microsomal cytochrome P450 content and ethylmorphine N-demethylase activity were reduced. Such effects were either less marked or absent in the mouse and Syrian hamster. Replicative DNA synthesis was studied by implanting osmotic pumps containing 5-bromo-2'-deoxyuridine during Study Weeks 0–1, 3–4, and 12–13. In the rat, coumarin administration for 4 and 13 weeks at dose levels of 0.3 and 0.5% produced a sustained stimulation of hepatocyte replicative DNA synthesis. No such effects were observed in the mouse and Syrian hamster. These results demonstrate marked species differences in coumarin-induced hepatotoxicity. While tumor formation in the rat appears due to chronic hepatotoxicity associated with a sustained regenerative hyperplasia, such effects were not observed in the CD-1 mouse and Syrian hamster. In assessing the hazard of coumarin to humans, account needs to be taken of both levels of exposure and species differences in response.

Coumarin (1,2-benzopyrone, cis-o-coumarinic acid lactone) is a white crystalline powder and occurs naturally in various plants and essential oils, including tonka beans, sweet clover, woodruff, and oils of cassia and lavender (Cohen, 1979). Although no longer used as a food flavoring, coumarin is present in certain tobaccos and alcoholic beverages and is used in various soap, detergent, and cosmetic preparations (Opdyke, 1974; Cohen, 1979).

The toxicology of coumarin merits attention because it has been shown to exhibit interspecies differences in both metabolism and toxicity (Cohen, 1979). While 7-hydroxylation is the major metabolic pathway in humans and certain primates, this represents only a minor pathway in other species, such as the rat, Syrian hamster, and most mouse strains (Shilling et al., 1969; Gangolli et al., 1974; Lush and Andrews, 1978; Cohen, 1979; Waller and Chasseaud, 1981; Rautio et al., 1992). The major route of coumarin metabolism in the rat is 3-hydroxylation which is followed by ring opening and further metabolism to other products including o-hydroxyphenylacetic acid (Kaghen and Williams, 1961; Cohen, 1979).

In the rat single doses of coumarin have been shown to produce centrilobular hepatic necrosis (Lake, 1984; Lake et al., 1989, 1994a), whereas chronic administration results in bile duct lesions (Hagan et al., 1967; Cohen, 1979; Evans et al., 1989). Both acute and chronic coumarin-induced hepatotoxicity may be due to the formation of a coumarin 3,4-epoxide intermediate (Lake, 1984; Lake et al., 1989, 1994a,b). The bile duct lesions produced by the chronic administration of coumarin have been described as cholangiitis, cholangioadenoma, and/or cholangiocarcinoma (Hagan et al., 1967; Grieppentrog, 1973; Cohen, 1979; Evans et al., 1989). While controversy exists as to the nature of the lesions produced by chronic coumarin administration, a threshold exists as toxicity and tumor formation were reported only at high (e.g., 0.5%) and not at low (e.g., 0.1%) dietary levels (Hagan et al., 1967; Cohen, 1979). The carcinogenicity of coumarin has been evaluated in a gavage study in F344 rats and B6C3F1 mice (NTP, 1993). Although coumarin at doses of up to 100 mg/kg did not produce liver tumors in F344 rats, some evidence of renal tubule adenoma was reported. Coumarin was reported to produce alveolar/
bronchiolar adenoma or carcinoma in B6C3F1 mice. In addition, in female mice significant increases in hepatocellular adenoma were reported at dose levels of 50 and 100 mg/kg, but not at the highest dose of 200 mg/kg (NTP, 1993).

The carcinogenicity of coumarin has also been examined in dietary studies conducted in Sprague-Dawley CD rats fed 0.0333–0.5% coumarin and CD-1 mice fed 0.03–0.3% coumarin (Carlton et al., 1996). Rats given 0.0333–0.2% coumarin (but not 0.3 and 0.5% coumarin) were exposed to these dose levels in utero, during the lactational period, and then chronically following weaning. No significant increases in tumors in the liver or in other tissues were observed in male and female CD-1 mice fed diets containing 0.03–0.3% coumarin (equivalent to intakes of 26–280 and 28–271 mg/kg/day in male and female animals, respectively). However, in male and female Sprague-Dawley CD rats high doses of coumarin did produce liver lesions and liver tumors (Carlton et al., 1996). Coumarin intakes in male rats fed 0.0333, 0.1, 0.2, 0.3, and 0.5% coumarin in the diet for 104 weeks were 13, 42, 87, 130, and 234 mg/kg/day, respectively. Increased liver weights were observed at the 0.3 and 0.5% dose levels and at the highest dose level an increased incidence of liver masses was noted at necropsy. An increased incidence of cholangiofibrosis, cholangiocarcinomas, and parenchymal cell tumors was observed in male rats fed 0.5% coumarin and a single cholangiocarcinoma in one rat given 0.3% coumarin was also considered to be potentially treatment related (Carlton et al., 1996). No significant increases in liver tumors were observed in rats given 0.02% coumarin in the diet. The incidences (n = 65 per group) of cholangiocarcinomas (both nonmetastasizing and metastasizing) were 0, 0, 0, 0, 1, and 37 and those of parenchymal cell tumors (both benign and malignant) were 2, 2, 1, 1, 6, and 29 in male rats fed 0.0, 0.0333, 0.1, 0.2, 0.3, and 0.5% coumarin, respectively (Carlton et al., 1996). In addition to the chronic studies in the rat and mouse, Ueno and Hirono (1981) have examined the carcinogenicity of coumarin in the Syrian hamster. No increase in bile duct proliferation, cholangiofibrosis, cholangiocarcinoma, or other tumors was observed in the livers of male and female Syrian hamsters fed 0.1 and 0.5% coumarin for up to 2 years (Ueno and Hirono, 1981).

The aim of the present study was to obtain further information on species differences in coumarin-induced hepatotoxicity. In addition to morphological and biochemical investigations, the effect of coumarin on hepatic replicative DNA synthesis was investigated, as several studies have suggested that increased cell replication may be an important factor in the carcinogenicity of certain chemicals (Butterworth, 1991; Cohen and Ellwein, 1991; Grasso et al., 1991; Ames et al., 1993). Male rats and mice of the strains used in the studies of Carlton et al. (1996) and male Syrian hamsters were fed various dietary levels of coumarin (Table 1) for 1, 4, and 13 weeks.

### MATERIALS AND METHODS

#### Chemicals.
Coumarin (98%) was obtained from Lancaster Synthesis Ltd. (Morecambe, Lancs, UK) and 5-bromo-2'-deoxyuridine (BRDU), enzyme cofactors, etc., were from Sigma-Aldrich Chemical Co. Ltd. (Poole, Dorset, UK).

#### Animals and treatment.
Male Sprague-Dawley CD rats and male CD-1 strain mice were obtained from Charles River UK Ltd. (Margate, Kent, UK) and male Syrian hamsters from Wrights of Essex Ltd. (Chelmsford, Essex, UK). Rats and mice were allowed free access to R and M No.1 diet (Special Diets Services, Witham, Essex, UK) and water, and Syrian hamsters were allowed free access to R and M No.3 diet and water. The animals were housed in groups of three to six depending on the species in mesh-bottomed cages in accommodations maintained at 22 ± 3°C with a relative humidity of 40–70% and were allowed to acclimatize to these conditions for at least 14 days before the study began. Rats (8 weeks old), mice (8 weeks old), and Syrian hamsters (7 weeks old) were fed diets containing no test compound (controls) or diets containing the concentrations of coumarin shown in Table 1 for periods of 1, 4, and 13 weeks. Animals were killed by exsanguination under diethyl ether anesthesia and the livers immediately excised for biochemical and morphological studies.

#### Replicative DNA synthesis.
Repetitive DNA synthesis was studied by subcutaneously implanting 7-day Alzet (Charles River UK Ltd., Margate, Kent, UK) osmotic pumps (rats, Model 2ML1; mice and hamsters, Model 2001) containing 15 mg/ml BRDU during Study Weeks 0–1, 3–4, and 12–13. Sections of liver and upper small intestine (to confirm pump efficiency) were fixed in neutral buffered Formalin. After the sections were processed, immunostaining of deparaffinized 5-μm sections was performed with an anti-BRDU antibody and 3,3′-diaminobenzidine (Barras et al., 1993). The hepatocyte labeling index (i.e., percentage of hepatocyte nuclei undergoing replicative DNA synthesis) was assessed by microscopic examination of at least 1000 nuclei in random fields from the left lobe.

#### Biochemical investigations.
Whole liver homogenates (rat 0.25 and mouse and Syrian hamster 0.1 g fresh tissue/ml) were prepared in ice-cold 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, using a Potter-type, Teflon-glass motor-driven homogenizer (A. H. Thomas Co., Philadelphia, PA). Whole homogenates were assayed for total glutathione (i.e., reduced GSH and oxidized (GSSG) glutathione) by an enzymatic recycling assay employing GSH reductase and 5,5′-dithio-bis (2-nitrobenzoic acid) (Adams et al., 1983) and for γ-glutamyltransferase activity employing L-γ-glutamyl-7-aminomethylcoumarin as substrate (Smith et al., 1979). Washed microsomal fractions were prepared by differential centrifugation and assayed for cytochrome P450 content and ethylmorphine N-demethylase activity (Lake, 1987). Protein was determined by the method of Lowry et al. (1951) employing bovine serum albumin as standard. Serum alanine aminotransferase activity and bilirubin content were determined with a Roche (Roche Products Ltd., Welwyn Garden City, Herts, UK) COBAS Mira reaction rate analyzer employing standard diagnostic test kits. The activity of serum γ-glutamyltransferase was determined by the method of Smith et al. (1979).

#### Morphological investigations.
Liver slices were fixed in neutral buffered Formalin. Paraffin sections of about 5-μm thickness were cut, stained with hematoxylin and eosin, and examined by light microscopy.

#### Statistical analysis.
Statistical evaluation of data was performed by one-way analysis of variance. Comparisons between means were made using the least significant difference test.

### RESULTS

**Compound intake and effect on food consumption.** Calculated daily intakes of coumarin are shown in Table 1. Owing to marked toxicity, rats treated with 0.75% coumarin were terminated after 7 weeks. Mean daily intakes of mice fed
0.05–0.5% coumarin for 13 weeks were some 2.2 to 2.6 times greater than those observed with corresponding dietary levels in the rat. However, mean daily intakes in rats fed 0.1–0.5% coumarin were 1.1 to 1.5 times greater than those obtained in the Syrian hamster. At the highest dose levels examined mean daily coumarin intakes in the mouse and Syrian hamster were 2.6 and 2.0 times greater, respectively, than those of rats fed 0.5% coumarin.

Food consumption was depressed in rats given 0.2–0.75% coumarin diets during the first 3 days of treatment. At longer treatment times food consumption was significantly reduced only in rats given 0.5 and 0.75% coumarin diets, being some 59 and 43% of control, respectively, over the treatment periods (data not shown). Food consumption in Syrian hamsters given 0.5 and 1.0% coumarin diets was reduced during the first 3 days of treatment, whereas no reduction in food consumption was observed in Syrian hamsters at longer treatment times or in mice given 0.05–0.5% coumarin at any treatment times (data not shown).

**Effect on body weight and relative liver weight.** Coumarin administration at dietary levels of 0.3–0.75% for 1 and 4 weeks and 0.3 and 0.5% for 13 weeks significantly reduced body weight gain in the rat (Fig. 1A). In addition, body weight gain was also reduced in rats given 0.2% coumarin for 1 and 13 weeks. Coumarin treatment at dietary levels of up to 0.5% had little effect on body weight gain in either the mouse (Fig. 1B) or Syrian hamster (Fig. 1C) and only produced a small reduction in body weight gain in Syrian hamsters given 1.0% coumarin for 13 weeks.

Treatment with 0.1% coumarin for 1 week produced a small increase in relative liver weight (i.e., liver weight/100 g body wt) in the rat (Fig. 1D). Significant increases in relative liver weight to 115–138% of control were observed in rats given 0.05–0.5% coumarin for 4 weeks and to 120–125% of control in rats given 0.05–0.2% coumarin for 13 weeks. Coumarin administration at certain dose levels produced small but statistically significant increases in relative liver weight in the mouse after 1, 4, and 13 weeks of treatment (Fig. 1E) and in the Syrian hamster after 13 weeks of treatment (Fig. 1F).

**Effect on serum parameters.** Serum samples were assayed for bilirubin content and activities of alanine aminotransferase and γ-glutamyltransferase. Significant increases in serum bilirubin levels were observed in rats given high doses of coumarin for 1, 4, and 13 weeks (Fig. 2A). A reduction in serum bilirubin levels was observed in mice at some coumarin dose levels after 1 and 4 weeks of treatment (Fig. 2B). Apart from a significant increase in animals given 1% coumarin for 1 week, coumarin treatment did not affect serum bilirubin levels in the Syrian hamster (Fig. 2C). Increases in serum alanine aminotransferase activity were observed at some coumarin dose levels and time points in both the rat (Fig. 2D) and mouse (Fig. 2E). Marked increases in serum alanine aminotransferase activity were observed in Syrian hamster given 0.5 and 1.0% coumarin for 1 week, but these increases were not sustained with prolonged treatment (Fig. 2F). Serum γ-glutamyltransferase activity was induced in the rat after 1, 4, and 13 weeks of treatment (Fig. 3A). In the mouse serum enzyme activity was increased in mice given 0.3% and/or 0.5% coumarin for 1 and 4 weeks, no data being available for 13 weeks (Fig. 3B). Only small changes in serum γ-glutamyltransferase activity were observed in the Syrian hamster (Fig. 3C).

**Effect on hepatic parameters.** Liver whole homogenates were assayed for γ-glutamyltransferase activity and total glutathione (i.e., GSH + GSSG) content. Significant dose-related increases in γ-glutamyltransferase activity were observed in rats given ≥0.1% coumarin for periods of 1, 4, and 13 weeks (Fig. 3D). In contrast, coumarin treatment had much less effect on γ-glutamyltransferase activity in the mouse (Fig. 3E) and essentially no effect in the Syrian hamster (Fig. 3F). Hepatic total glutathione content was assayed at some coumarin treatment levels in all three species after 1 and 13 weeks. In the rat, coumarin treatment produced significant increases in total glutathione content, particularly after 13 weeks of treatment (Table 2). Additional studies demonstrated that the increase in total glutathione was primarily due to increased GSH levels, with very little effect being observed on GSSG levels (data not shown). In the mouse treatment with 0.3 and 0.5% coumarin reduced total glutathione content, whereas apart from an increase in animals given 1.0% coumarin for 1 week, no effect was observed in the Syrian hamster (Table 2).

The effect of coumarin treatment on microsomal xenobiotic metabolism was monitored by measuring total cytochrome P450 content and ethylmorphine N-demethylase activity. In the rat, high doses of coumarin significantly re-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Dietary level (%)</th>
<th>Rat (mg/kg/day)</th>
<th>Mouse (mg/kg/day)</th>
<th>Syrian hamster (mg/kg/day)</th>
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<tr>
<td>0.05</td>
<td>41.1</td>
<td>91.1</td>
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<tr>
<td>0.1</td>
<td>83.0</td>
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<tr>
<td>0.2</td>
<td>156</td>
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</tr>
<tr>
<td>0.3</td>
<td>253</td>
<td>560</td>
<td>197</td>
</tr>
<tr>
<td>0.5</td>
<td>346</td>
<td>895</td>
<td>231</td>
</tr>
<tr>
<td>0.75</td>
<td>486</td>
<td>—</td>
<td>677</td>
</tr>
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</table>

* Calculated from body weight and food consumption data on Study Days 0–3, 14–17, 42–45, and 70–73.
* Due to toxicity rats receiving 0.75% coumarin were terminated after 7 weeks.
Produced both cytochrome P450 content (Fig. 4A) and ethylmorphine N-demethylase activity (Fig. 4D) after 1, 4, and 13 weeks of treatment. Some decreases in cytochrome P450 content (Fig. 4B) and ethylmorphine N-demethylase activity (Fig. 4E) were observed in the mouse after 4, but not after 1 and 13 weeks of treatment. In contrast, cytochrome P-450 content was not significantly affected by coumarin treatment in the Syrian hamster (Fig. 4C) and only a small effect was observed on ethylmorphine N-demethylase activity in animals fed 0.1% coumarin for 13 weeks (Fig. 4F).

Effect on liver morphology. Histopathological changes (Fig. 5) occurred in rats at all three time points and they were particularly marked after 13 weeks of treatment. The principal hepatic change observed in rats killed after 1 week (Table 3) consisted of fine vacuoles which morphologically were consistent with fatty change. The affected cells were randomly scattered throughout the liver but were more noticeable around the portal vein. They showed no dose-response relationship. Similar vacuoles were present in controls but were much less numerous than in treated animals.

FIG. 1. Effect of feeding diets containing 0 (control, □), 0.05 (□), 0.1 (□), 0.2 (□), 0.3 (□), 0.5 (□), 0.75 (□), and 1.0 (□) % coumarin for periods of 1, 4, and 13 weeks to rats (A, D), mice (B, E), and Syrian hamsters (C, F) on body weight (A, B, C) and relative liver weight (D, E, F). Results are expressed as means ± SE of five to eight animals. Values significantly different from control are *<p < 0.05, **p < 0.01, ***p < 0.001.
A few apoptotic cells were observed but their incidence did not appear to be any more marked than that expected in the livers of untreated rats. In the group fed 0.75% coumarin in the diet, centrilobular cell enlargement and cell necrosis (Fig. 5A) was observed in some animals (Table 3).

The most prominent lesions observed after 4 weeks in rats given ≥0.2% coumarin consisted of necrosis and vacuolar degeneration and were present in most of the lobules examined. The necrotic cells occurred centrilobularly and were observed in three of four rats treated with 0.5 and 0.75% coumarin (Table 3). These cells were swollen, had lost most of their cytoplasm, and had a small pyknotic nucleus. Vacuolar degeneration, a manifestation of intracellular edema, was observed in the centrilobular and midzonal areas in all animals fed 0.75% coumarin and in three of four animals fed 0.5% coumarin (Table 3). The affected cells were larger than normal and contained varying amounts of finely granular eosinophilic material which occupied most of the cell, the cytoplasm being displaced toward the periphery of the cell. Centrilobular cell necrosis was also observed in rats given
0.3% coumarin and vacuolar degeneration in rats given 0.2 and 0.3% coumarin. The lesions observed at these two dose levels were much milder than those observed at the higher treatment levels. Apoptosis and fatty change were also observed in the livers of rats treated with 0.3–0.75% coumarin for 4 weeks (Table 3). The cells affected were more numerous than in controls and the effects appeared to be dose related. While fatty change appeared to be mainly centrilobular, apoptosis was scattered throughout the liver lobule. Bile duct hyperplasia and cholangiofibrosis were also observed after 4 weeks of coumarin treatment. It was very mild and affected two of four and three of four animals fed 0.5 and 0.75% coumarin, respectively. Very few bile ducts were affected.

After 13 weeks, the principal changes in rats fed 0.5% coumarin consisted of periportal cell enlargement, vacuolar degeneration (Fig. 5B), bile duct hyperplasia, and cholangiofibrosis (Figs. 5D and 5E). The bile duct hyperplasia and cholangiofibrosis were prominent, and the other lesions less so (Table 3). No convincing evidence of hepatocellular ne-
crosis was present. Vacular degeneration and an increase in apoptosis (Fig. 5C) was observed in rats fed 0.2–0.5% and 0.3 and 0.5% coumarin, respectively, but there was no evidence of any liver damage in rats fed 0.05 and 0.1% coumarin (Table 3).

In contrast to the rat, no histopathological changes attributable to coumarin treatment were observed in either the mouse or the Syrian hamster.

**Effect on replicative DNA synthesis.** Replicative DNA synthesis was determined over Study Weeks 0–1, 3–4, and 12–13 using 7-day osmotic pumps to continuously administer BRDU. After 1 week replicative DNA synthesis, assessed as the hepatocyte labeling index, was significantly increased to 292–365% of control levels in rats given 0.05-0.3% coumarin (Fig. 6A). Hepatocyte labeling index values were also significantly increased after 1 week of treatment to 218, 680, and 1803% of control in mice (Fig. 6B) given 0.1, 0.3, and 0.5% coumarin, respectively, and to 464% of control in Syrian hamsters (Fig. 6C) given 1.0% coumarin.

In the rat the administration of 0.3 and 0.5% coumarin, but not 0.05–0.2% coumarin, resulted in a sustained elevation of labeling index values to 619 and 1213% of control after 4 weeks and to 826 and 1546% of control after 13 weeks, respectively (Fig. 6A). Labeling index values were also increased to 1506% of control in rats given 0.75% coumarin for 4 weeks. Coumarin treatment for 4 weeks did not produce any significant increase in labeling index values in either the mouse (Fig. 6B) or the Syrian hamster (Fig. 6C). Apart from a small, but not dose-related, increase in animals given 0.3% coumarin, no significant effects on replicative DNA synthesis were observed in these two species after 13 weeks of treatment.

**DISCUSSION**

Previous studies have demonstrated that the acute administration of coumarin to rats produces centrilobular hepatic necrosis, whereas prolonged administration results in bile duct lesions (Hagan et al., 1967; Griepentrog, 1973; Cohen, 1979; Lake, 1984; Evans et al., 1989; Lake et al., 1989, 1994a,b). Evidence for dose-response relationships has also been obtained and controversy exists as to the nature of the bile duct lesions (Hagan et al., 1967; Griepentrog, 1973; Cohen, 1979; Evans et al., 1989; Carlton et al., 1996).

In the present study coumarin produced clear dose-related hepatotoxic effects in the rat. The principal pathological changes consisted of vacuolar degeneration (ballooning, intracellular edema), necrosis and apoptosis of hepatocytes, and proliferative changes in the bile ducts. The vacuolar changes had a marked zonal distribution and are likely to represent intracellular accumulation of water due to failure of the osmotic pump mechanism. Lesions of this sort may lead to autolytic necrosis (Toner et al., 1992). Apoptosis, a different form of cell death, showed no zonal distribution but had a clear dose-related response suggesting that it was causally related to coumarin administration. Originally apoptosis was thought to represent only ‘‘programmed’’ cell death but it is now recognized that it may also be a manifestation of lethal cell injury (Alison and Sarraf, 1995). Bile duct hyperplasia and cholangiofibrosis were first noted at 4 weeks. At this time point the hepatic lesions had not shifted to the periportal zone so that the bile duct lesions cannot be regarded as a secondary effect of periportal cell necrosis as they are in the periportal lesion induced by linear short chain esters of allyl alcohol (Bar and Griepentrog, 1967). A direct effect of coumarin on the small bile duct cells is likely, even though no damage could be seen in these cells by light microscopy.

At dietary levels of 0.3 and 0.5% where liver tumors were observed in male Sprague-Dawley CD rats by Carlton et al. (1996), coumarin produced marked hepatotoxic effects as demonstrated by the changes in the serum (e.g., bilirubin), morphological, and biochemical parameters measured. To provide a sensitive measure of changes in hepatocyte replicative DNA synthesis (Goldsworthy et al., 1991), animals were implanted with 7-day osmotic pumps containing BRDU. A sustained increase in hepatocyte labeling index values after
4 and 13 weeks of treatment was paralleled by increases in mitosis and/or apoptosis, necrosis, and vacuolar degeneration (Table 3).

A variety of studies have demonstrated that cell proliferation is an important factor in the development of carcinogenesis by both genotoxic and nongenotoxic chemicals (Butterworth, 1991; Cohen and Ellwein, 1991; Goldsworthy et al., 1991; Weisburger, 1994). Coumarin has been reported to be negative in a number of short-term tests for mutagenic and genotoxic potential, although equivocal or weakly positive results have also been obtained in other studies (Cohen, 1979; NTP, 1993). Cell replication may lead to tumor formation through a variety of mechanisms not involving primary genomic damage. For example, an enhanced rate of DNA replication could increase the frequency of spontaneous mutations and the probability of converting DNA adducts from both exogenous and endogenous sources into mutations before they can be repaired (Butterworth, 1991; Goldsworthy et al., 1991; Ames et al., 1993). In general there are two broad classes of chemicals which have been found to induce
cell replication in rodent liver. The first class comprises mitogenic agents which produce additive hyperplasia with little or no toxicity, whereas the second class comprises cytotoxic agents where cell proliferation is the result of a regenerative hyperplasia (Loury et al., 1987; Goldsworthy et al., 1991; Grasso and Hinton, 1991; Ames et al., 1993). Examples of such rodent liver cytotoxic agents include carbon tetrachloride, chloroform, and furan (Loury et al., 1987; Grasso et al., 1991; Wilson et al., 1992; Larson et al., 1994).

While it is claimed that liver carcinogenesis is not always associated with induced hepatocyte proliferation (Melnick and Huff, 1993), the data obtained in the present study demonstrate a very good correlation between sustained cell replication and subsequent tumor formation (Fig. 7). At dietary...
<table>
<thead>
<tr>
<th>Parameter</th>
<th>1 week</th>
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<th>13 weeks</th>
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<tr>
<td></td>
<td>Control</td>
<td>0.05</td>
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<tr>
<td></td>
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<tr>
<td></td>
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</tr>
<tr>
<td>Dietary level (%)</td>
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<td>Vacuoles (fat)</td>
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<tr>
<td>Vacuolar degeneration (ballooning)</td>
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<tr>
<td>Clear cell (glycogen)</td>
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</table>

Severity of liver lesions were classified as 0, no lesion; >0-1, mild; >1-2, moderate; and >2-3, severe. Figures shown are mean values with the number of animals exhibiting the lesion shown in parentheses. The figures in square brackets indicate the range of severity of the lesion in the animals examined.

Mitosis was scored as cells in metaphase.
levels of ≤0.2% coumarin does not increase the incidence of either parenchymal cell tumors or cholangiocarcinoma, whereas at higher doses (0.3 and 0.5%) the sustained stimulation of replicative DNA synthesis is associated with tumor formation (Fig. 7). As such the present data with coumarin are similar to those obtained in studies with furan (Wilson et al., 1992). Furan has been shown to produce hepatotoxic effects in the rat, a stimulation of hepatocyte labeling index values, and chronic administration results in hepatocellular adenomas/carcinomas and cholangiocarcinomas (Wilson et al., 1992).

While the precise mechanism of coumarin-induced hepatotoxicity in the rat awaits elucidation, evidence has been obtained that toxicity is due to a coumarin 3,4-epoxide intermediate generated by cytochrome P-450 isoenzymes and that GSH can protect against hepatocyte injury (Lake, 1984; Lake et al., 1989, 1994a,b; den Besten et al., 1990; Fentem et al., 1992). In agreement with previous studies coumarin treatment resulted in the induction of hepatic γ-glutamyltransferase activity and increases in GSH content (Lake et al., 1990, 1994b). Coumarin treatment has also been shown to increase GSH S-transferase activities and it is likely that the elevation in serum γ-glutamyltransferase activity is due to induction of hepatic enzyme activity (Siest et al., 1988; Lake et al., 1994b). The changes in GSH content and activities of GSH S-transferase and γ-glutamyltransferase may be attributed to enhanced detoxification of a coumarin 3,4-epoxide and possibly other reactive intermediates by formation of GSH conjugates. Coumarin is known to enhance urinary mercapturic acid excretion in the rat (Lake, 1984) and a coumarin mercapturic acid metabolite has been identified in rat urine (Huwer et al., 1991).

Another aspect of coumarin-induced hepatotoxicity which merits further study is the shift of the site of hepatic damage from the centrilobular to the periportal region of the liver lobule after acute and chronic administration, respectively. This may be due to a number of factors. For example, the site of acute coumarin-induced hepatic necrosis in rat liver can be modulated by pretreatment with known inducers of cytochrome P450 isoenzymes (Lake and Evans, 1993). While coumarin administration reduces total hepatic cytochrome P450 content and certain mixed-function oxidase enzymes, such effects may not be uniform across the liver lobule. Another possible explanation is that the bile duct lesions may be attributable to local toxicity caused by the breakdown of unstable coumarin metabolites (e.g., GSH conjugates).

Although coumarin produced marked hepatotoxicity in the rat, it was clearly much less toxic in either the CD-1 mouse or the Syrian hamster. Coumarin administration at compound intake levels which exceeded those administered to the rat (Table 1) had no marked effect on body weight in the mouse and Syrian hamster (Fig. 1). Although some
increases in serum bilirubin levels and alanine aminotransferase activity were observed these were not associated with any significant effects on liver morphology. Compared to the rat, coumarin administration to the mouse and Syrian hamster had much less effect on serum and hepatic $\gamma$-glutamyltransferase activities and hepatic microsomal cytochrome P450 content and ethylmorphine $N$-demethylase activity (Figs. 3 and 4). Unlike that in the rat, coumarin treatment had little effect on total glutathione levels in the Syrian hamster and produced a reduction at high dose levels in the mouse (Table 2). The effects of coumarin on the markers of phase I xenobiotic metabolism, total glutathione content, and $\gamma$-glutamyltransferase activity in the mouse and Syrian hamster compared to the rat suggest that differences in the pathways of coumarin metabolism and/or disposition may exist in these two species. A major difference between the rat and the other two species studied was the effect of coumarin administration on hepatocyte replicative DNA synthesis. While coumarin treatment for 1 week did produce some increases in the hepatocyte labeling index in the mouse and Syrian hamster, there was no evidence for a sustained stimulation of cell replication in these two species (Fig. 6). The increase in replicative DNA synthesis at high coumarin doses in these two species was not associated with any observable increase in necrosis and/or apoptosis.

The lack of chronic coumarin-induced hepatotoxicity in the CD-1 mouse and Syrian hamster is in agreement with previous studies conducted in these species (Ueno and Hirono, 1981; Carlton et al., 1996). However, in a gavage study coumarin was reported to produce a non-dose-related increase in hepatocellular adenomas in female, but not male, B6C3F1 mice (NTP, 1993). The reason for this apparent anomaly between the two chronic mouse studies might be due to mouse strain differences, but is more likely due to pharmacokinetic differences. Under bioassay conditions, significantly higher peak coumarin levels in mouse plasma and liver were observed after gavage in B6C3F1 mice as against dietary administration in CD-1 mice (personal communication from Research Institute for Fragrance Materials, Inc., New Jersey, USA).

In summary, these results demonstrate marked species differences in coumarin-induced hepatotoxicity. Chronic administration of high doses to the rat results in a sustained regenerative hyperplasia of parenchymal and bile duct cells which is associated with the formation of both parenchymal cell tumors and cholangiocarcinomas. In agreement with previous studies in the rat, clear dose-response relationships for coumarin-induced liver damage are observed. While coumarin is hepatotoxic in the rat at doses which markedly affect body weight gain, it is clearly much less toxic in the CD-1 mouse and Syrian hamster. Furthermore, coumarin did not produce a sustained stimulation of replicative DNA synthesis in these two species. In assessing the hazard, if any, of coumarin to humans account should be taken of both levels of exposure and species differences in response. Little evidence of hepatotoxicity has been reported in humans given 25–400 mg/day coumarin in clinical trials to treat various malignancies and chronic infections (Cox et al., 1989; Dexeus et al., 1990). Such data suggest that coumarin is unlikely to be hepatotoxic in humans under normal levels of exposure (e.g., from soap, detergent, and cosmetic preparations). Thus in assessing the significance of coumarin-induced hepatotoxicity, the mouse and Syrian hamster appear to be better models for man than the rat. With these former two species clear no effect levels were observed and even in the rat, the susceptible species, a clear threshold exists before significant hepatotoxicity is observed.

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