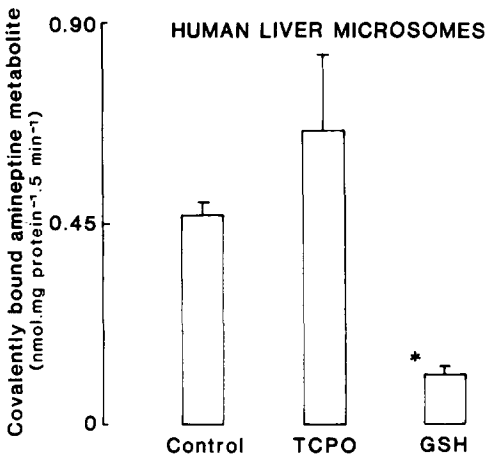

Fig. 3. Effects of glutathione and an inhibitor of epoxide hydrolase on *in vitro* covalent binding with human liver microsomes. In some flasks, 0.1 mM of 1,1,1-trichloropropene 2,3-oxide (TCPO) or 4 mM of reduced glutathione (GSH) were added to the incubation mixture. Results are means ± SEM for six determinations. The asterisk indicates a significant difference from the value in the control incubation ($P < 0.01$).

tine has the clinical features of an allergic phenomenon [3, 4]. Thus, hepatitis occurs after repeated doses, appearing 10 days to 6 months after the onset of the treatment; hepatitis is usually associated with hypersensitivity manifestations, and promptly recurs after a rechallenge. Hypothetically, the metabolite-macromolecule complexes may, in a few immunologically predisposed subjects, lead to immunization against the drug-altered macromolecules, resulting in an allergic type of hepatitis [6].

We conclude that amineptine is transformed by human cytochrome P-450 into a reactive metabolite which is efficiently detoxified by glutathione or may covalently bind to hepatic proteins.

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Microsomal cholesterol epoxide hydrolase activity in 2-acetylaminofluorene-induced rat liver hyperplastic nodules

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Cholesterol 5,6-oxide formed endogenously from lipid-peroxidation-mediated epoxidation of cholesterol [1] has been shown to possess mutagenic and carcinogenic properties [2]. This compound is formed in the skin of mice upon exposure to ultraviolet light [3] and also causes malignant transformation of Chinese hamster cells in culture [4]. Cholesterol epoxide hydrolase, which hydrolyzes cholesterol 5,6-oxide to cholestan 3β,5α,6β-triol, thus has an important role in the detoxification of this epoxide. This enzyme in rat and mouse liver microsomes is distinct from the well studied microsomal epoxide hydrolase (EC 3.3.2.3) that hydrolyzes several arene and alkene oxides [5, 6]. Hyperplastic nodules induced in rat liver by chronic feeding of 2-acetylaminofluorene and believed to be the precursors for the later stages of hepatocellular carcinoma contain 3- to 4-fold elevated levels of microsomal epoxide hydrolase (assayed using styrene oxide as substrate) [7]. A number of heterocyclic compounds such as harman, norharman, ellipticine, isoquinoline, metyrapone, chalcone, and 9-fluorenone have been reported to be potent stimulators of microsomal styrene oxide hydrolase activity [8, 9], and the degree of stimulation with these compounds has been found to be greater with the enzyme from hyperplastic nodules [9]. No reports are available on the level of cholesterol epoxide hydrolase in hyperplastic nodules and how the enzyme is affected by the compounds that stimulate styrene oxide hydrolase. In the present study, hyperplastic liver nodules were found to contain reduced levels of cholesterol epoxide hydrolase activity, and the various stimulators of styrene oxide hydrolase inhibited cholesterol epoxide hydrolysis by microsomes from control and hyperplastic nodules.

Materials and methods

[4-^{14}C]Cholesterol (sp. act. 54 mCi/mmol) was obtained from New England Nuclear, Boston, MA. Harman, norharman, isoquinoline, metyrapone, 9-fluorenone, chalcone, 1,1,1-trichloropropene oxide and acetonitrile

were obtained from the Aldrich Chemical Co., Milwaukee, WI. Ellipticine was a gift from the National Cancer Institute. LK5D Linear-K silica gel plates were purchased from Whatman Laboratory Products Inc., Clifton, NJ. Tetrahydrofuran and scintillation fluid were from the Eastman Kodak Co., Rochester, NY. Methylene chloride was obtained from the Baker Chemical Co., Phillisburg, NJ. All other chemicals used were of analytical grade.

[4-^{14}C]Cholesterol 5,6-oxide was prepared as follows. [4-^{14}C]Cholesterol was diluted to a specific activity of 10 mCi/mmol with unlabeled cholesterol. After evaporation of benzene from cholesterol under N_2, *m*-chloroperbenzoic acid (1.6 mg) in 1 ml of methylene chloride was added along with 10 mg of anhydrous sodium carbonate. The reaction mixture was stirred overnight after which no trace of unreacted cholesterol could be detected by thin-layer chromatography. The excess peracid was decomposed by vigorous mixing with 1 N sodium thiosulfate, followed by a wash with saturated sodium carbonate. After separation of the organic phase, the aqueous phase was extracted once with 1 ml methylene chloride. The organic phases were combined and evaporated under N_2, then taken up in 1 ml benzene, and applied to silica gel column (2 g) equilibrated in benzene. The column was eluted successively with 10 ml of benzene and 8 ml of benzene–diethylether (3:1, v/v). Fractions of 2 ml were collected and analyzed by thin-layer chromatography using diethylether as solvent (cholesterol, $R_f = 0.85$; cholesterol 5,6-oxide, $R_f = 0.57$). Cholesterol 5,6-oxide was detected in the first two fractions of the second elution solvent with 98% chemical purity and 94% radiopurity. After evaporation of the solvent, cholesterol epoxide was taken in acetonitrile. Cholestan 3β,5α,6β-triol was synthesized according to the method of Fieser and Rajagopalan [10] and squalene oxide according to the method of Nadeau and Hanzlik [11].

Male Sprague–Dawley rats weighing 100–150 g were obtained from SASCO (Omaha, NE) and were fed basal diet [12]. Hyperplastic liver nodules were produced by the method of interrupted feeding of 0.05% 2-acetylaminofluorene for 15 weeks in basal diet [13]. Microsomes from control livers and hyperplastic nodules were prepared by homogenization in 250 mM sucrose, 24 mM KCl, 5 mM $MgCl_2$, and 5 mM Tris–HCl, pH 7.4, followed by differential centrifugation [9]. The twice washed microsomal pellets were suspended in the above buffer by gentle homogenization.

Cholesterol 5,6-oxide hydrolase activity was measured according to the method of Levin *et al.* [6]. The assay mixture contained, in a final volume of 80 μl, 100 mM potassium phosphate buffer, pH 7.4, 125 μM [4-^{14}C]cholesterol 5,6-oxide (1 mCi/mmol) and microsomes (100–150 μg protein). After incubation at 37° for 15 min, the reaction was stopped by the addition of 25 μl of tetrahydrofuran containing 10 μg of cholestan 3β,5α,6β-triol. Thin-layer chromatographic separation of cholestanetriol was carried out using the solvent system, tetrahydrofuran, methylene chloride (8:2, v/v) and was identified by exposure to iodine vapour. The radioactivity in the spot corresponding to authentic cholestanetriol was measured. Activity was expressed as nmol of cholestan 3β,5α,6β-triol formed per min per mg protein. The heterocyclic compounds were made water soluble by tituration with glacial acetic acid and were added at a final concentration of 1 mM in dilute acid. 9-Fluorenone, chalcone, and 1,1,1-trichloropropene oxide were dissolved in acetonitrile, and final concentration of the solvent in the reaction mixture was maintained at 6.25% [6]. Protein was measured according to the method of Lowry *et al.* [14].

Results and discussion

Cholesterol 5,6-oxide hydrolase activity was assayed under the conditions where the activity was proportional to time and to microsomal protein concentration. The microsomes from control liver showed an enzyme activity of 0.17 nmol of cholestanetriol formed per min per mg protein. A decrease of about 60% in cholesterol 5,6-oxide hydrolase activity was observed in the microsomes from hyperplastic liver nodules compared to controls fed basal diet (Table 1). The kinetics of this decrease in enzyme activity in hyperplastic nodules were determined from Lineweaver–Burk plots (Fig. 1). The enzyme showed the same K_m value of 91 μM as in controls. A decrease in V_{max} of the enzyme (0.07 nmol cholestanetriol formed/min/mg protein), however, was observed compared to the enzyme from control liver, thus indicating a reduction in enzyme content rather than a change in characteristics of the enzyme in hyperplastic nodules. In contrast to cholesterol 5,6-oxide hydrolase activity, microsomal styrene oxide hydrolase activity was reported to be elevated 3- to 4-fold in hyperplastic nodules compared to control livers [7]. The reduction in cholesterol 5,6-oxide hydrolase in microsomes of hyperplastic nodules which parallels the decreased cyto-

Table 1. Effects of certain heterocyclic compounds on microsomal cholesterol 5,6-oxide hydrolase activity from control liver and hyperplastic nodules

	Cholesterol 5,6-oxide hydrolase activity (nmol cholestan 3β,5α,6β-triol formed/min/mg protein)	
Compound*	Control	Hyperplastic nodules
None	0.17 ± 0.001†	0.07 ± 0.006‡
Harman	0.1 ± 0.01§	0.03 ± 0.003‖
Norharman	0.13 ± 0.01§	0.03 ± 0.006§
Ellipticine	0.02 ± 0.003‖	0.004 ± 0.001‖
Metyrapone	0.17 ± 0.01	0.047 ± 0.006§
Isoquinoline	0.15 ± 0.03	0.047 ± 0.006§
9-Fluorenone	0.15 ± 0.001§	0.053 ± 0.003§
Chalcone	0.08 ± 0.01§	0.02 ± 0.004§

* All compounds except ellipticine were present at 1 mM concentration. Ellipticine was added at 0.5 mM concentration.

† Mean ± SEM of three separate determinations.

‡ $P < 0.0005$, analysis for significance (*t*-test) was made by comparing control and hyperplastic nodules.

§ $P < 0.005$, and ‖ $P < 0.0005$, analyses for significance (*t*-test) were made by comparing the effect of addition of compound with the control in each group.

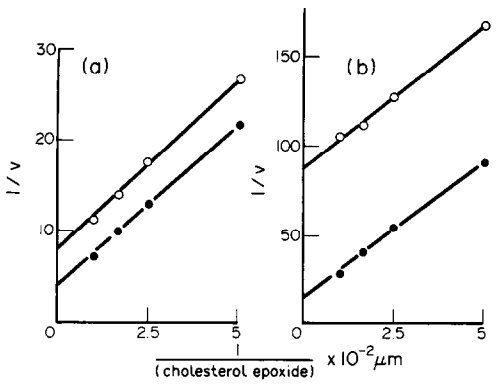

Fig. 1. Lineweaver–Burk plots for inhibition of cholesterol 5,6-oxide hydrolase activity from control liver (a) and hyperplastic nodules (b) by ellipticine (100 μM). Key: (●——●) without ellipticine, and (○——○) with ellipticine.

chrome P-450 levels [15] indicates that similar control mechanisms may exist for both of these enzyme systems in rat liver. Though the significance for the reduction in cholesterol 5,6-oxide hydrolase activity is not understood, it may lead to the accumulation of cholesterol 5,6-oxide in the nodules. This epoxide was shown to bind to DNA covalently forming adducts [16]. Oesch [17] proposed that covalent binding of epoxides to DNA leads to mutagenicity and to the initiation of cancer. Cholesterol 5,6-oxide was also reported to cause chromosomal damage and induce DNA repair processes in human fibroblasts [18]. Sevanian and Peterson [19] reported that the mutagenicity of cholesterol 5,6-oxide to V79 Chinese hamster cells is abolished by its conversion to cholestanetriol. Thus, the available studies suggest the possibility that cholesterol 5,6-oxide may be involved in chemical hepatocarcinogenesis.

The effects of the heterocyclic compounds known to stimulate styrene oxide hydrolase [8, 9] were tested on cholesterol 5,6-oxide hydrolase activity from both control and hyperplastic nodule microsomes, and the results are shown in Table 1. Ellipticine, chalcone, harman, norharman, and 9-fluorenone significantly inhibited cholesterol 5,6-oxide hydrolase activity from nodules and control liver. A greater degree of inhibition by these compounds was observed for the enzyme from nodules compared to control. Ellipticine, the most potent stimulator of styrene oxide hydrolase from nodules and control liver [9], was found to be the most potent inhibitor of cholesterol 5,6-oxide hydration. The inhibition observed with control and nodule microsomes at 0.5 mM concentration of the compound was 88 and 95% respectively. The next highest inhibition was observed with chalcone, and this compound inhibited 53% of the enzyme activity from control and 71% of the activity from hyperplastic nodules. Metyrapone and isoquinoline at 1 mM concentration had no significant effect on the enzyme from control liver, whereas an inhibition of about 33% was observed with the enzyme from hyperplastic nodules. An inhibitory effect by these compounds on the enzyme from control liver (inhibition of 26 and 24% respectively) was observed, however, at 2.5 mM concentration. 1,1,1-Trichloropropene oxide, the potent inhibitor of styrene oxide hydrolase [20] and monooxygenase activities [21] in rat liver microsomes, was shown to have no effect on cholesterol epoxide hydrolase activity in mouse liver microsomes [5] but rather stimulates the enzyme from bovine liver microsomes [22]. The enzyme from hyperplastic nodules or control rat liver was unaffected by this compound. The greater degree of inhibition observed by the heterocyclic compounds on the enzyme from hyperplastic nodules could be due to altered membrane properties of the microsomes isolated from nodules as reported by Griffin and Kizer [7] and by Erikson *et al.* [23]. Some of the heterocyclic compounds tested, like metyrapone and ellipticine, were shown to inhibit microsomal monooxygenase system [8, 24]. Since ellipticine produced the highest degree of inhibition of cholesterol 5,6-oxide hydrolase activity, the kinetics of inhibition of the compound were studied (Fig. 1). An uncompetitive type of inhibition by ellipticine was observed for the enzyme from control liver or hyperplastic nodules, showing that the inhibitor binds to enzyme–substrate complex. The binding resulted in a decrease in V_{max} of the enzyme from control liver and hyperplastic nodules by 43 and 86% respectively. The K_m values of the enzyme decreased from 91 to 48.5 μM in control liver and to 18.6 μM in hyperplastic nodules (Fig. 1). Thus, cholesterol 5,6-oxide hydrolase from the preneoplastic stage of hepatocarcinogenesis appears to be affected drastically by its inhibitors compared to enzyme from control liver.

In summary, microsomal cholesterol 5,6-oxide hydrolase activity from hyperplastic nodules of rats induced by feeding 2-acetylaminofluorene was decreased significantly compared to controls fed basal diet. The enzyme from hyperplastic nodules had the same K_m (91 μM) as the control but showed a decreased V_{max}, suggesting a reduced amount of the enzyme in hyperplastic nodules. The known heterocyclic stimulators of styrene oxide hydrolase inhibited cholesterol 5,6-oxide hydrolase activity, and the highest inhibition was observed with ellipticine in both control and hyperplastic nodules. The enzyme from hyperplastic nodules was inhibited to a greater extent by these compounds than the enzyme from control. An uncompetitive type of inhibition by ellipticine was observed for the enzyme from control or hyperplastic nodules.

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