Differential Induction of Cytosolic Epoxide Hydrase, Microsomal Epoxide Hydrolase, and Glutathione S-Transferase Activities

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Differential Induction of Cytosolic Epoxide Hydrase, Microsomal Epoxide Hydrolase, and Glutathione S-Transferase Activities. HAMMOCK, B. D. AND OTA, K. (1983). Toxicol. Appl. Pharmacol. 71, 254–265. Three major enzyme systems have been shown to metabolize epoxidized xenobiotics in vertebrate tissues, and this study demonstrates that these enzyme systems can be differentially induced. The cytosolic epoxide hydrase activity was routinely monitored with trans-β-ethylstyrene oxide, the microsomal epoxide hydrolase activity with benzo(a)pyrene, 4,5-oxide, and the glutathione S-transferase activity with 2,4-dichloro-4-nitrobenzene. Commonly used inducers of microsomal mixed-function oxidase, microsomal epoxide hydrolase, and cytosolic glutathione S-transferase activities failed to cause significant induction of the cytosolic epoxide hydrolase while leading to the expected induction of the other epoxide metabolizing enzymes. The compounds tested by ip injection into male Swiss-Webster mice included phenobarbital, 3-methylcholanthrene, Arochlor 1254, trans- and cis-stilbene oxides, pregnenolone-16α-carbonitrile, chalcone, and 4-bromochalcone. To determine if there were strain, sex, or species differences, the enzymes were monitored in male C57BL/6 mice, female Swiss-Webster mice, and male Sprague-Dawley rats following ip injection of phenobarbital, 3-methylcholanthrene, and/or pregnenolone-16α-carbonitrile. The time dependence of enzyme induction was followed in Sprague-Dawley rats following trans-stilbene oxide administration. Male Swiss-Webster mice were additionally exposed to dietary α-naphthoflavone and 2(3)-tert-butyl-4-hydroxyanisole while male Sprague-Dawley rats were fed 2,6-di-tert-butyl-4-methylphenol. In no case was significant induction of cytosolic epoxide hydrase activity observed. Dietary di(2-ethylhexyl)phthalate, 2-ethyl-1-hexanol, and clofibrate proved to be potent inducers of the cytosolic epoxide hydrolase in male Swiss-Webster mice while probucol (a nonperoxisome proliferating hypolipidemic drug) failed to cause significant induction. Data from isoelectric focusing experiments and other data are consistent with the epoxide hydrolase activities induced by 2-ethyl-1-hexanol and clofibrate being due to the same protein that is present in control animals. The lack of induction of the cytosolic epoxide hydrolase by a variety of compounds which were selected to demonstrate induction of other xenobiotic metabolizing enzymes, may indicate that the cytosolic epoxide hydrolase has a constitutive role whereas its induction by clofibrate could be related to some of the pharmacological and/or carcinogenic actions of this drug.

Many synthetic and naturally occurring epoxidized compounds (epoxides) exist in the environment or arise in vivo by the enzymatic oxidation of olefinic or aromatic compounds. Epoxides vary dramatically in their chemical reactivity and in their affinity for biological molecules. Though many epoxides are biodegradable, biologically innocuous compounds, at the extremes of reactivity they may be environmentally persistent materials which accumulate in the food chain such as dieldrin or electrophilically reactive agents such as arene oxides which can alkylate biological nucleophiles leading to toxic, mutagenic, or carcinogenic effects. An understanding of the balance between epoxide formation and deg-
radation is important for an appreciation of their environmental fate and their fate and action in biological systems. Certainly with reactive epoxides an appreciation of their relative rates of formation and detoxification is important for risk assessment.

Epoxides appear to be metabolized by three major enzymes in vertebrate liver. Glutathione S-transferases (EC 2.5.1.18) catalyze the nucleophilic attack of the sulfur anion of glutathione (GSH) on the epoxide to yield the corresponding conjugate (Chassaud, 1979). Epoxide hydrolases (EHs) (EC 3.3.2.3) catalyze the addition of water to the epoxide moiety to yield a 1,2-diol (Oesch, 1973; Lu and Miwa, 1980). As indicated in the above reviews, the predominantly cytosolic GSH S-transferases and predominantly microsomal EH(s) have been investigated extensively. However, for many substrates the cytosolic–mitochondrial EH is far more active than the microsomal form. The cytosolic and microsomal EHs appear to be distinct enzymes which differ in their substrate selectivity; susceptibility to inhibitors; species, sex, tissue, and subcellular distribution; pH optima and numerous biochemical parameters including distinct antigenic determinants, molecular weight, subunit composition, and isoelectric points (Hammock et al., 1980a,b; Mullin and Hammock, 1980, 1982; Oesch and Golan, 1980; Ota and Hammock, 1980; Gill and Hammock, 1981a,b; Guenther et al., 1981). Thus, it is of interest to see if the cytosolic EH can be induced by the same compounds which induce the microsomal EH and GSH S-transferase activities. In this study induction refers to a change in enzyme activity in a crude liver fraction in response to a xenobiotic. No attempt was made in this study to prove that a compound fails to induce the cytosolic EH. However, by monitoring the levels of GSH S-transferase and microsomal EH as positive controls, it is possible to determine if the cytosolic epoxide hydrolase is regulated differently from the other two epoxide metabolizing systems. Analogous studies have demonstrated, for instance, the differential control of the microsomal EH and mixed-function oxidase activities (Oesch, 1976; Schmassmann and Oesch, 1978). Since differential induction of activating and deactivating enzymes has been shown in several laboratories to influence the mutagenicity and/or carcinogenicity of xenobiotics, such information is of critical importance.

METHODS

Chemicals. cis-Stilbene oxide (CSO) and trans-β-ethyl styrene oxide (TESO) were synthesized by peracid oxidation of the appropriate isomerically pure olefin with m-chloroperbenzoic acid (Mullin and Hammock, 1980), 4-bromocyclohexanol [1-phenyl-3(4-bromophenyl)-2-propene-1-one] was synthesized by the condensation of 4-bromobenzaldehyde and acetophenone in base (Mullin and Hammock, 1982). Clofibrate (ethyl α-[4-chlorophenoxy]-α-methylpropionate, ECPIB, clofibrate, CPIB, ICI-28297, Astromid-S, Regelan) was synthesized according to the procedure of Bach (1970). trans-Stilbene oxide (TSO), cis-stilbene, 4-bromobenzaldehyde, acetophenone, α-naphthol, naphthalene, 1,3-diphenyl-2-propen-1-one, 2-ethyl-1-hexanol (sec-octanol), and butylated hydroxytoluene (2,6-di-tert-buty1-4-methylphenol, BHT) were purchased from Aldrich Chemical Co. (Milwaukee, Wisc.), 3-methylcholanthrene (3-MC) was from Eastman Chemical Co. (Rochester, N.Y.), trans-1-phenyl-2-butanone from Chemical Samples (Columbus, Ohio), glutathione (GSH) from Sigma Chemical Co. (St. Louis, Mo.), and di(2-ethylhexyl) phthalate (DEHP) from ICN/K & K Chemicals (Plainview, N.Y.). Aroclor 1254, phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), and 2,4-dichloro-4-nitrobenzene were provided by R. Talcott of the University of California (San Francisco, Calif.). Probufol [4,4′-isopropylidenediathio]bis(2,6-di-tert-buty1phenol) was received gratis from Dow Chemical Co. (Indianapolis, Ind.).

Radiochemicals. [3H]-β-Ethyl styrene oxide was synthesized by the method of Mullin and Hammock (1980) and diluted with unlabeled TESO to a specific activity of ca. 1 mCi/mmol. [3H]benzo(a)pyrene-4,5-oxide (BPO) (specific activity ca. 1 mCi/mmol) was a generous gift from F. Oesch of the University of Mainz, West Germany.

Treatment of animals. Male and female Swiss–Webster mice (8 weeks old at the time of treatment, males 25 to 30 g, females 20 to 25 g), male C57BL/6 mice (8 weeks old, 20 to 25 g), and male Sprague-Dawley rats (6 weeks old, 120 to 150 g for phenobarbital and 7 weeks old for 130 to 220 g for 3-MC) were purchased from Simonsen Labs (Gilroy, Calif.) and were housed in plastic cages with pine shavings for bedding. They were allowed free access to standard rodent chow or specialized diet and tap water up to the time of sacrifice.

Doses and treatment schedules were selected based upon successful literature procedures when available. Animals
receiving PB, 3-MC, TSO, CSO, PCN, chalcone, or 4-
bromochalcone were treated between 9 and 11 AM on 3
successive days by an ip injection of the compound in
an appropriate vehicle and were killed 24 hr after the final
treatment. Animals treated with ArcoÞ 1254 were injected
once ip and killed 3 days later. PB was injected in 0.1 ml
(mouse) and 1.0 ml (rat) of physiological saline, PCN was
injected in 0.1 ml of propylene glycol, and all other com-
ounds were injected in 0.1 ml (mouse) and 1.0 ml (rat)
of corn oil. Control animals were treated with the vehicle
only.

DEHP, clofibrate, and 2-ethyl-1-hexanol were dissolved
in corn oil and mixed into ground rodent chow at a ratio
of 10 ml corn oil solution per 100 g chow. Final percentages
of DEHP, clofibrate, and 2-ethyl-1-hexanol were 2, 0.5,
and 2% (w/w), respectively. Control animals received a
corn chow mixture at the same ratio of oil to chow.

Probenecid, naphthoﬂavone, and BHT diets were prepared
by mixing the crystals into ground chow at a concentration
of 0.5%. Animals receiving 2,3-ent-butyl-4-hydroxyani-
side (BHA) were fed a pelleted diet consisting of BHA at
0.75% (w/w) which was kindly provided by A. Poland of
the University of Wisconsin (Madison, Wisc.). Control
animals received standard rodent chow. Experimental an-
imals with the exception of BHA animals remained on
their diets for 10 days, and BHA animals were on their
diets for 8 days.

Preparation of subcellular fractions. Animals were killed
without prior fasting beginning at 9 AM by cervical dis-
location and their livers dissected free of adhering tissues
and weighed. The livers were perfused in vitro with ice
cold 1.15% KCl to remove blood. All subsequent pro-
cedures were done at 0 to 4°C. Livers were homogenized
individually in phosphate bufer (pH 7.4, 76 mm) with a
Tekmar Tissumizer at setting 30 for 15 sec. The homog-
genates were then centrifuged at 10,000g for 20 min, and
the resulting supernatant fractions were centrifuged at
100,000g for 75 min. The supernatant fraction remaining
(cytoisol) was pipetted into vials with care taken to mini-
imize contamination by lipids. The microsomal pellets
were resuspended in the same volume of bufer used for
homogenization and frozen at −70°C until assayed. Cyto-
isol specimens not assayed within 24 hr of preparation
were also frozen at −70°C. These procedures resulted in
minimal losses of activity. Although microsomes prepared
in this fashion were contaminated with trapped cytosol,
only minimal hydrolysis of BPO by the cytosolic EH was
observed, corroborating the results of Oesch and Golan
(1980). No additional marker enzymes were used to de-
termine the influence of xenobiotics on the recovery or
purity of the microsomal fraction.

Enzyme and protein assays. The cytosolic EH activities
were determined by the radiometric partition procedure
of Mulin and Hammock (1980), with the substrate (TESO)
at a final concentration of 0.5 mm. Only the cytosolic
form of the enzyme was examined.

Microsomal EH activities were determined by a mod-
ification of the method of Jerina et al. (1977). Microsomes
were diluted in Tris-HCl bufer (pH 9.0, I = 0.1) and
150 µl of the resultant dilution was preincubated at 37°C
for about 1 min. An acetonirole solution (2 µl) of BPO
was delivered to give a final substrate concentration of
0.05 mm. The reaction was stopped by the addition of
100 µl of ether, followed by mixing. A small amount of
NaCl was then added and the sample was mixed again.
An additional 100 µl of ether was then added before the
samples were centrifuged to break emulsions. This ex-
traction procedure resulted in the recovery of >95% of
the radioactivity. The ether layer was then spotted on the
cellulose pretayer of Whatman 1,000F silica gel thin-layer
plates (Clifton, N.J.) and developed with toluene:methanol
(9:1) as the solvent system. The plates were visualized
under uv light and the spots corresponding to BPO and
the resulting diol were scraped into vials. Scintillation
bufer was then added to the vials and the samples were
allowed to sit overnight in the dark before counting by
liquid scintillation. On occasion the microsomal epoxide
hydrodase activity was also monitored with 3H-labeled CSO
at a substrate concentration of 0.05 mm by either the th-
inner procedure described above or a partition assay (Mul-
in and Hammock, 1982).

Cytoisol GSH S-transferase activities (subsequently
referred to as GSH-transferase) were assayed according
to the procedure of Habig et al. (1974) with 1,2-dichloro-4-
nitrobenzene as the second substrate at a final concen-
tration of 1 mm and a GSH concentration of 5 mm. Fresh
GSH solutions were made for each experiment. Rates
were measured as the increase in absorption at 345 nm
with a Cary 219 or Beckman 20 spectrophotometer. No
attempt was made to distinguish enzyme patterns or to
examine the microsomal form of the enzyme.

Protein levels were determined by modifications of the
Lowry method as suggested by Benadou and Weinstein
(1976). Statistical treatment of the results was by Student’s
t test. Signiﬁcance was assumed at α = 0.05.

Cytosols from control, clofibrate- and 2-ethyl-1-hexa-
ol-treated animals were analyzed by slab-gel isoelectric
focusing on a LKB Multiphor apparatus. The samples
were analyzed ﬁrst by wide range and then by narrow
range (Ampholine, pH 4 to 6) isoelectric focusing (Winter
et al., 1977). The gel tracks were sliced (5 mm) and eluted
for 12 hr in phosphate bufer (0.5 mm) before analysis for
EH activity. The pH gradient was determined by slicing
the gel (1 cm) and eluting the slices in 1 ml of glass distilled
water. The pH was then measured at 5°C with a Corning
476050 electrode standardized with pH 4 and 7 American
Scientific Products’ bufers (McGaw Park, Ill.).

RESULTS

Eight compounds were first examined for their ability to induce epoxide metabolizing
enzymes following ip administration to male
Swiss–Webster mice (Table 1). This study was then extended with three compounds to examine the effects of species, sex, and strain on induction (Table 2). Finally, the induction of these enzymes was examined following oral administration of seven additional compounds (Table 3). None of these compounds shown in Tables 1 and 3 caused significant induction of the cytosolic EH in male Swiss–Webster mice while they led to the expected increases in the other enzyme systems. PB and Arochlor 1254 led to a statistically significant (31%) decrease in the cytosolic EH activity and a significant increase in the other enzymes. 3-MC only caused a significant increase in GSH S-transferase levels. The lack of induction of the microsomal EH by 3-MC and the poor induction by PB (which shows significance only at the $\alpha = 0.1$ level) corroborates the results obtained by Oesch et al. (1973) for the Swiss–Webster strain of mouse. Neither TSO or CSO caused significant induction of the cytosolic EH while TSO led to large increases in the microsomal EH and GSH S-transferase levels. CSO led to significant decrease in microsomal EH while proving to be a moderately effective inductor of GSH S-transferase activity. Interestingly, it appeared to only be able to induce GSH S-transferase 2-fold while TSO induced it 3.2-fold. In male mice PCN caused no change in the cytosolic EH, little change in the microsomal EH, and a 1.8-fold increase in GSH S-transferase activity. As shown in Table 3 dietary $\alpha$-naphthoflavone led to a decrease in the cytosolic EH and an increase in the other enzymes. As expected, dietary BHA led to a 6.5-fold increase in the microsomal EH and an 8-fold increase in GSH S-transferase activities while reducing the cytosolic EH levels to 56% of control levels. The more toxic BHT had a similar effect in the rat.

The results obtained when the study was extended to other strains, sexes, and species are shown in Table 2. In no case was significant induction of the cytosolic EH observed at $\alpha = 0.05$. Although no significant induction of the cytosolic EH is observed for the C57BL/6 strain, the large decrease in cytosolic EH activity observed with PB treatment of Swiss–Webster mice is not observed with C57BL/6 mice. In fact, there was no significant difference between control and treated mice. PB induced both microsomal EH and GSH S-transferase 1.4- and 2.0-fold, respectively, but no significant induction of either of these enzymes was observed with 3-MC treatment. Since adult male Swiss–Webster mice have much higher levels of hepatic cytosolic EH (Gill and Hammock, 1980), it was considered possible that the male could be in a permanently induced state and the female in an uninduced state. However, female Swiss–Webster mice showed no significant induction of cytosolic EH after PB or 3-MC treatment. Again with female Swiss–Webster mice the significant reduction of cytosolic EH activity after PB treatment was not observed. A further observation is that the cytosolic EH activity in male Swiss–Webster mice after PB induction is lowered to approximately the same level as that found in uninduced female Swiss–Webster mice of the same age. PCN caused little or no change in the cytosolic EH of either male or female mice; it induced GSH S-transferase 1.8-fold in both cases, while it was slightly more potent in inducing the microsomal EH in male than male mice (1.9- vs 1.3-fold). Rats treated with PB and 3-MC showed significant induction of the microsomal EH by PB but not by 3-MC (Table 2). Both compounds significantly induce GSH S-transferase. 3-MC failed to induce the cytosolic EH while PB led to a slight ($\alpha = 0.10$) increase in the cytosolic EH.

Another possibility is that induction of the cytosolic and microsomal EHs is coupled, but the EHs occur on a different time scale. Following a single ip administration of 2 mmol/kg body weight TSO to male rats, microsomal EH reached peak levels on Day 2 followed by a slow decline over a 5-day period. GSH S-transferase activity followed a similar course. No significant change in cytosolic EH activity was observed during this period (data not shown).

The study was then extended to several
<table>
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<tr>
<th>Compound</th>
<th>Dosage (mg/kg)</th>
<th>Cytosolic* epoxide hydrolase (nmol/min/mg protein)</th>
<th>Microsomal* epoxide hydrolase (nmol/min/mg protein)</th>
<th>Glutathione* transferase (nmol/min/mg protein)</th>
<th>Liver weight (g)</th>
<th>Cytosolic protein (mg/liver)</th>
<th>Microsomal protein (mg/liver)</th>
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<tr>
<td>Control</td>
<td>0</td>
<td>62.3 ± 12.0</td>
<td>5.55 ± 0.64</td>
<td>16.9 ± 4.0</td>
<td>1.91 ± 0.19</td>
<td>7.65 ± 8.7</td>
<td>15.3 ± 0.3</td>
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<td>PB</td>
<td>40</td>
<td>53.6 ± 12.0</td>
<td>6.42 ± 2.60</td>
<td>19.4 ± 5.2</td>
<td>2.03 ± 0.12</td>
<td>48.4 ± 4.7*</td>
<td>20.6 ± 2.9*</td>
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<td>3-MC</td>
<td>20</td>
<td>58.3 ± 14.8</td>
<td>6.90 ± 1.69</td>
<td>25.6 ± 7.8*</td>
<td>2.07 ± 0.34</td>
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<td>51.9 ± 16.6</td>
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<td>52.0 ± 11.2**</td>
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<td>2.58 ± 0.11</td>
<td>10.8 ± 13*</td>
<td>36.7 ± 8.9*</td>
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<td>TSO</td>
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<td>4-Bromochalcone</td>
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<td>70.6 ± 11.5</td>
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<td>2.20 ± 0.12</td>
<td>131 ± 4*</td>
<td>28.0 ± 1.1</td>
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* For assay conditions see Methods. Results represent mean ± standard deviation for hepatic tissues from six mice treated and prepared individually.

* Significantly different from the control animals (α = 0.05).

** Significantly different from the control animals (α = 0.1).
<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Sex</th>
<th>Compound</th>
<th>Dosage (mg/kg)</th>
<th>Cytosolic&lt;sup&gt;*&lt;/sup&gt; epoxide hydrolase (nmol/min/mg protein)</th>
<th>Microsomal&lt;sup&gt;*&lt;/sup&gt; epoxide hydrolase (nmol/min/mg protein)</th>
<th>Glutathione&lt;sup&gt;*&lt;/sup&gt; transferase (nmol/min/mg protein)</th>
<th>Liver weight (g)</th>
<th>Cytosolic protein (mg/liver)</th>
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<td>1.32 ± 0.15</td>
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<td>68.3 ± 9.0</td>
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<tr>
<td>Mouse</td>
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<td>PB</td>
<td>80</td>
<td>46.6 ± 10.0</td>
<td>5.48 ± 1.21&lt;sup&gt;**&lt;/sup&gt;</td>
<td>25.3 ± 1.1</td>
<td>1.79 ± 0.21&lt;sup&gt;**&lt;/sup&gt;</td>
<td>63.3 ± 7.5</td>
<td>32.8 ± 4.4&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Mouse</td>
<td>SW Female</td>
<td>Control</td>
<td>0</td>
<td>39.4 ± 2.4</td>
<td>4.25 ± 0.78</td>
<td>17.2 ± 1.7</td>
<td>1.69 ± 0.13</td>
<td>60.8 ± 16.0</td>
<td>17.8 ± 2.6</td>
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<td>Mouse</td>
<td>SW Female</td>
<td>3-MC</td>
<td>40</td>
<td>38.8 ± 7.4</td>
<td>5.01 ± 0.59&lt;sup&gt;**&lt;/sup&gt;</td>
<td>34.4 ± 6.1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.84 ± 0.16</td>
<td>63.6 ± 7.3</td>
<td>22.0 ± 3.5&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Mouse</td>
<td>SW Female</td>
<td>Control</td>
<td>0</td>
<td>29.0 ± 5.2</td>
<td>2.13 ± 0.77</td>
<td>12.4 ± 4.1</td>
<td>1.50 ± 0.22</td>
<td>61.1 ± 12.0</td>
<td>16.4 ± 1.3&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
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<td>PCN</td>
<td>20</td>
<td>31.2 ± 7.3</td>
<td>4.00 ± 1.37&lt;sup&gt;**&lt;/sup&gt;</td>
<td>22.8 ± 4.3&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.57 ± 0.22</td>
<td>67.7 ± 9.3</td>
<td>20.6 ± 2.8&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Rat</td>
<td>SD Male</td>
<td>Control</td>
<td>0</td>
<td>4.54 ± 0.35</td>
<td>16.0 ± 5.0</td>
<td>16.3 ± 3.3</td>
<td>5.71 ± 1.36</td>
<td>235 ± 28</td>
<td>61.9 ± 7.3</td>
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<tr>
<td>Rat</td>
<td>SD Male</td>
<td>PB</td>
<td>80</td>
<td>6.46 ± 2.40&lt;sup&gt;**&lt;/sup&gt;</td>
<td>24.6 ± 6.1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>24.7 ± 7.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>5.03 ± 0.89</td>
<td>234 ± 15</td>
<td>83.6 ± 8.2&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Rat</td>
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<td>0</td>
<td>7.01 ± 1.28</td>
<td>17.7 ± 4.2</td>
<td>19.8 ± 4.7</td>
<td>9.41 ± 0.41</td>
<td>466 ± 6</td>
<td>179 ± 5</td>
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</tbody>
</table>

<sup>*</sup> For assay conditions see Methods. Results represent mean ± standard deviation for hepatic tissues from six rodents treated and prepared individually.

<sup>**</sup> Significantly different from the control animals (α = 0.10).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (% in diet, w/w)</th>
<th>Cytosolic&lt;sup&gt;a&lt;/sup&gt; epoxide hydrolase (nmol/min/mg protein)</th>
<th>Microsomal&lt;sup&gt;a&lt;/sup&gt; epoxide hydrolase (nmol/min/mg protein)</th>
<th>Glutathione S-transferase&lt;sup&gt;a&lt;/sup&gt; (nmol/min/mg protein)</th>
<th>Liver weight (g)</th>
<th>Cytosolic protein (mg/liver)</th>
<th>Microsomal protein (mg/liver)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>71.4 ± 8.4</td>
<td>8.91 ± 2.12</td>
<td>16.0 ± 2.8</td>
<td>2.02 ± 0.15</td>
<td>90.9 ± 10.3</td>
<td>17.1 ± 2.2</td>
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<tr>
<td>α-Naphthyllavine</td>
<td>0.2%</td>
<td>48.8 ± 9.9*</td>
<td>12.6 ± 2.1*</td>
<td>37.2 ± 8.8*</td>
<td>2.17 ± 0.28</td>
<td>104 ± 8*</td>
<td>18.2 ± 3.3</td>
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<tr>
<td>(10 days)</td>
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</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>72.1 ± 10.2</td>
<td>6.91 ± 0.56</td>
<td>9.44 ± 2.60</td>
<td>2.23 ± 0.08</td>
<td>87.3 ± 12.1</td>
<td>23.2 ± 4.0</td>
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<tr>
<td>BHA</td>
<td>0.75%</td>
<td>40.6 ± 5.0*</td>
<td>45.1 ± 5.6*</td>
<td>76.0 ± 27.8*</td>
<td>2.35 ± 0.25</td>
<td>112 ± 21*</td>
<td>30.8 ± 1.3*</td>
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<tr>
<td>(8 days)</td>
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<tr>
<td>Control</td>
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<td>5.79 ± 0.54</td>
<td>16.4 ± 3.1</td>
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<td>12.7 ± 1.2</td>
<td>571 ± 12</td>
<td>206 ± 19</td>
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<tr>
<td>BHT (Sprague–Dawley rat)</td>
<td>0.5%</td>
<td>3.92 ± 1.54</td>
<td>70.3 ± 15.4*</td>
<td>40.4 ± 3.1*</td>
<td>14.8 ± 0.9</td>
<td>684 ± 44*</td>
<td>295 ± 18*</td>
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<tr>
<td>(10 days)</td>
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</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>89.9 ± 19.6</td>
<td>6.91 ± 0.56</td>
<td>11.0 ± 2.8</td>
<td>2.03 ± 0.17</td>
<td>108 ± 23</td>
<td>24.7 ± 0.7</td>
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<tr>
<td>DEHP</td>
<td>2%</td>
<td>140 ± 14.2*</td>
<td>15.0 ± 1.5</td>
<td>28.6 ± 3.0*</td>
<td>3.45 ± 0.42*</td>
<td>236 ± 30*</td>
<td>59.6 ± 5.8*</td>
</tr>
<tr>
<td>(10 days)</td>
<td></td>
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<tr>
<td>Control</td>
<td>0</td>
<td>71.4 ± 8.4</td>
<td>8.91 ± 2.12</td>
<td>16.0 ± 2.8</td>
<td>2.02 ± 0.15</td>
<td>90.9 ± 10.3</td>
<td>17.1 ± 2.2</td>
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<tr>
<td>2-Ethyl-1-hexanol</td>
<td>2%</td>
<td>140 ± 13*</td>
<td>19.6 ± 4.1</td>
<td>27.2 ± 7.3*</td>
<td>2.40 ± 0.39*</td>
<td>115 ± 8.3*</td>
<td>20.8 ± 2.5*</td>
</tr>
<tr>
<td>(10 days)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>68.1 ± 18.8*</td>
<td>3.45 ± 0.55*</td>
<td>28.9 ± 4.9</td>
<td>1.94 ± 0.20</td>
<td>33.4 ± 2.3</td>
<td>28.4 ± 0.6</td>
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<tr>
<td>Clofibrate</td>
<td>0.5%</td>
<td>174 ± 6*</td>
<td>7.66 ± 0.51*</td>
<td>20.4 ± 2.5*</td>
<td>2.47 ± 0.24*</td>
<td>150 ± 13*</td>
<td>44.1 ± 3.7*</td>
</tr>
<tr>
<td>(10 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>81.4 ± 8.4</td>
<td>5.27 ± 0.88</td>
<td>—</td>
<td>2.43 ± 0.37</td>
<td>113 ± 20</td>
<td>17.4 ± 1.1</td>
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<tr>
<td>Probucol</td>
<td>0.5%</td>
<td>77.6 ± 7.7</td>
<td>5.08 ± 1.89</td>
<td>—</td>
<td>2.41 ± 0.17</td>
<td>107 ± 2</td>
<td>19.7 ± 1.3*</td>
</tr>
</tbody>
</table>

<sup>a</sup> For assay conditions see Methods. Results represent mean ± standard deviation for hepatic tissues from six rodents treated and prepared individually.

<sup>b</sup> In a separate experiment cytosolic EH levels were found to be 74.5 ± 7.6 and 177 ± 16 nmol/min/mg protein with TESO and 5.38 ± 0.48 and 13.7 ± 0.9 nmol/min/mg protein for TSO in control and induced mice, respectively. Microsomal EH levels were found to be 6.99 ± 0.47 and 14.0 ± 0.7 with BPO and 4.03 ± 0.63 and 9.21 ± 1.67 with CSO in control and induced mice, respectively.

<sup>*</sup> Significantly different from the control animals (α = 0.05).
compounds not classically used as inducers. Chalcone and 4-bromochalcone have been examined as stimulators and inhibitors of epoxide hydration and GSH conjugation (Ganu and Alworth, 1978; Mullin and Hammock, 1982; unpublished data). When they were examined as inducers, they had no effect on the cytosolic EH. However, they were marginal inducers of the microsomal EH and good inducers of GSH S-transferase. The total hepatic GSH S-transferase activity increased three-fold following chalcone treatment.

In contrast to all other compounds previously tested, DEHP led to a large, 1.6-fold increase in the cytosolic EH activity. Due to the large increase in liver size and protein content, this value represented a 3.4-fold increase in total liver cytosolic EH activity. DEHP was also a potent inducer of the microsomal EH and GSH S-transferase leading to a 2.1- and 2.6-fold increase in specific activity, respectively. With regard to the enzyme activities, liver weight, and protein content, 2-ethyl-1-hexanol caused similar responses to DEHP.

Although clofibrate is structurally unrelated to DEHP, it elicits many responses which are very similar to those following DEHP administration including reduction of serum cholesterol and triglyceride levels, peroxisome proliferation, and hepatomegaly. As with DEHP, a dramatic increase in enzyme activities was observed with clofibrate. A 2.6-fold increase in the specific activity and a 4-fold increase in total activity were observed with the cytosolic EH. There is the possibility that a different form of the cytosolic EH is induced following drug treatment. Purification studies to date, however, have failed to show multiple forms of the cytosolic EH in mouse or rabbit. Analytical isoelectric focusing demonstrated that the cytosolic EH activity from control, 2-ethyl-1-hexanol-, and clofibrate-treated animals cofocused with a single pI of approximately 5.4. This technique could readily detect differences in pI of 0.1 pH unit. Similarly as indicated in a footnote to Table 3 the relative increase in the specific activity following clofibrate treatment is very similar when each of two different substrates is used for the cytosolic and microsomal EH. Undoubtedly multiple forms of GSH S-transferase are involved in the composite activities reported in Tables 1–3.

Since DEHP, clofibrate, and 2-ethyl-1-hexanol elicit similar effects on hepatic morphology and on lipid metabolism, a fourth compound, probucol, was tested as a possible inducer of the cytosolic EH. This compound does not cause the hepatomegaly and the proliferation of peroxisomes observed with DEHP, clofibrate, and 2-ethyl-1-hexanol. However, significant decrease in serum cholesterol and lipid levels is observed upon administration of this drug to mice, rats, and monkeys (Barnhart et al., 1970) similar to that of clofibrate. To investigate the possibility that the increased cytosolic EH activities might be due in part to the associated decrease of cholesterol biosynthesis, probucol was administered to mice for 10 days. The results in Table 3 show that no significant induction of the microsomal or cytosolic EH activity was observed.

**DISCUSSION**

The major goal of this study was to test the hypothesis that the cytosolic EH levels in hepatic tissue are regulated differently from two other enzyme systems involved in epoxide metabolism, the microsomal EH and GSH S-transferases. None of the classically employed inducers of xenobiotic metabolism which were screened in this study induced the cytosolic EH. Mice exposed to the food additive BHA gave the most unequivocal demonstration that the hypothesis of differential regulation is correct. Dietary antioxidants have been shown to increase the specific activities of a variety of drug metabolizing enzymes as well as to reduce the carcinogenicity, toxicity, and/or DNA binding of several xenobiotics (Wattenberg, 1972; Weisburger et al., 1977; Cha et al., 1978; Benson et al., 1979; Cha and Bueding, 1979; Kahl, 1980; Benson et al., 1980; Salocks et al., 1981 and included references). As expected the microsomal EH and GSH S-
transferase activities increased 6.5- and 8-fold, respectively, while in this study the specific activity of the cytosolic EH dropped to 56% of the control values. This decrease in the specific activity of the cytosolic EH cannot be totally accounted for by the increase in total cytosolic protein. Because of this differential induction, BHA could be a very useful tool for investigating the relative role of drug-metabolizing enzymes. Since BHA is a relatively nontoxic food additive that is rapidly metabolized in vertebrate systems, it could prove useful in enhancing the clearance of oxidized xenobiotics from domestic animals following accidental exposure.

Although not as dramatic as BHA, all of the other commonly used inducers also demonstrated differential regulation of the epoxide metabolizing enzymes. The selective induction of some xenobiotic metabolizing enzymes by TSO and related compounds has been well documented (Mukhtar et al., 1978; Schmassmann and Oesch, 1978; Schmassmann et al., 1978; Guthenberg et al., 1980). As reported by Oesch and Schmassmann (1979), the induction of the microsomal EH by TSO is much more pronounced in the rat than in the mouse. The data shown in Table 1 are in line with these previous observations. Oesch and Schmassmann (1979) raised the possibility of differential metabolism, but ultimately concluded that TSO receptor affinity was a likely hypothesis to explain the different levels of induction in rats and mice. The high turnover of TSO by the mouse cytosolic EH (Hammock et al., 1980a,b; Oesch and Golan, 1980) may also serve to explain the lower induction and lower toxicity observed in the mouse (Oesch and Schmassmann, 1979). TSO proved to be a more potent inducer of GSH S-transferase in the mouse than has been previously observed in the rat (Mukhtar et al., 1978) and tends to support the observations of Viviani et al. (1980). CSO is hydrated by the microsomal EII faster than it is hydrated by the cytosolic EH (Oesch et al., 1971; Hammock et al., 1980a,b) but it is very rapidly degraded by GSH S-transferases. Thus, its lack of induction of the cytosolic EH was not surprising, but the unexpected decrease observed in the microsomal EH levels may prove useful.

The male Swiss-Webster mouse may not prove to be an appropriate model for induction studies. However, similar data were obtained with C57BL/6 mice. Although in the same “genotype” with respect to the pH optimum and heat stability of the microsomal EH (Lyman et al., 1980), the C57BL/6 mice were chosen as an alternative strain to the Swiss-Webster strain due to its responsiveness to mixed-function oxidase induction by 3-MC (Nebert et al., 1975; Poland and Kende, 1977) and microsomal EH induction by PB (Oesch et al., 1973). Also the cytosolic EH from C57BL/6 mice were shown to have the highest specific activity of all strains examined to date (Gill and Hammock, 1980). Other experiments with female Swiss-Webster and Sprague-Dawley rats supported the original conclusions regarding differential induction.

The hypothesis that enzyme inhibitors should be good inducers certainly did not hold in the case of the cytosolic EH and the two chalcones tested. However, these compounds represent novel structures which do induce GSH S-transferase and the microsomal EH. They are also inhibitors of some GSH S-transferase activity in addition to being depleters of GSH.

DEHP is of toxicological interest because it and related solvents and plasticizers are major industrial products. Due largely to their widespread use in polyvinyl chloride plastics (where they may account for up to 40% of the plastic’s total weight), phthalate esters are pervasive in man’s diet and environment (Peakall, 1975). DEHP was first examined in this study based upon the observations of Lake et al. (1975) that DEHP administration to rats led to mitochondrial swelling and peroxisome proliferation, and the observation by Gill and Hammock (1981a) of significant EH activity in the mitochondrial–peroxisomal subcellular fractions from mouse liver. Since 2-ethyl-1-
hexanol is a major metabolite of DEHP in several species including primates (Peakall, 1975; Albro et al., 1981), and it has been shown to account for many of the hepatic changes induced by DEHP (Lake et al., 1975; Moody and Reddy, 1978), it was also examined in this study.

Clofibrate and related compounds are commonly prescribed drugs due to their hypolipidemic action. They share this activity as well as their propensity to induce peroxisome proliferation and hepatomegaly in rodents with DEHP (Azarnoff et al., 1965; Reddy et al., 1976; Moody and Reddy, 1978). Thus it was not surprising that clofibrate also shared the property of inducing the cytosolic EH. Since both DEHP and clofibrate have hypolipidemic action and among other things inhibit hepatic squalene and sterol biosynthesis (Azarnoff et al., 1965; Witiak et al., 1969; Bell, 1976; Suzuki, 1976), it was crucial to test another hypolipidemic agent not known to induce peroxisomal proliferation or hepatomegaly. The fact that probucol (Barnhart et al., 1970) failed to induce the cytosolic EH indicates that such an induction is not necessary for hypolipidemic action in general.

The hypolipidemic action of DEHP, clofibrate, and related compounds is undoubtedly due to numerous factors. However, induction of the cytosolic EH may play some role in this hypolipidemic action. Some oxygenated sterols and their precursors such as squalene 2,3-24,25-dioxide and lanosterol 24,25-epoxide are known to be potent angiototoxic agents (Imai et al., 1980). These highly lipophilic, trisubstituted epoxides are excellent substrates for the cytosolic EH (Hammock et al., 1980a). If such oxygenated sterols are direct angiototoxic agents or are involved in cholesterol regulation (Kandutsch et al., 1978), induction of the cytosolic EH could have direct hypolipidemic effects. The cytosolic EH is also known to hydrate rapidly lipid epoxides such as trans- and cis-epoxysteranes (Gill and Hammock, 1979). Based upon structure activity relationships, the cytosolic EH is likely to hydrate potential chemical mediators such as epoxidized precursors of the prostaglandin pathway which may influence indirectly lipid levels and distributions.

Regardless of speculative pharmacological roles, DEHP, 2-ethyl-1-hexanol, and clofibrate will be useful in conjunction with other inducers and inhibitors in elucidating the roles of these enzymes in the metabolism and action of epoxidized xenobiotics. Hopefully further research will lead to still more selective and potent tools.

REFERENCES


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HAMMOCO AND OTA


