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Epoxide Metabolism in the Liver of Mice Treated with Clofibrate (Ethyl-α-(p-chlorophenoxyisobutyrate)), a Peroxisome Proliferator[1]

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Epoxide Metabolism in the Liver of Mice Treated with Clofibrate (Ethyl-α-(p-chlorophenoxyisobutyrate)), a Peroxisome Proliferator. MOODY, D. E., LOURY, D. N., AND HAMMOCK, B. D. (1985). *Toxicol. Appl. Pharmacol.* **78,** 351–362. An increase in cytosolic epoxide hydrolase (cEH) activity occurs in the livers of mice treated with peroxisome proliferating–hypolipidemic–nongenotoxic carcinogens. As increases in activity of epoxide metabolizing enzymes may reflect the carcinogenic mechanism, a detailed comparison of the response of cEH, microsomal epoxide hydrolase (mEH), and cytosolic glutathione *S*-transferase (cGST) activities using the geometrical isomers *trans*- and *cis*-stilbene oxide as substrates has been performed in livers from mice treated with clofibrate (ethyl-α-(p-chlorophenoxyisobutyrate)). The maximal increase of cEH activity occurred at lower dietary doses of clofibrate (0.5%) and within a shorter time (5 days) than mEH and cGST (2%, 14 days) activity. After 14 days at 0.5% clofibrate, cEH, mEH, and cGST activities were 250, 175, and 165% and 290, 220, and 75% of control values in male and female mice, respectively. Withdrawal of clofibrate from the diet resulted in a reversion of activities to control values within 7 days. Clofibrate treatment shifted the apparent subcellular compartmentation of all three enzymatic activities with an increase in the ratio of soluble to particulate activity. In particular, the relative specific activity of all three enzymes decreased in the light mitochondrial (peroxisomal) cell fraction, and an increase of a mEH-like activity (benzo[*a*]pyrene-4,5-oxide and *cis*-stilbene oxide hydrolysis) in the cytosol occurred. Both the increase of cEH activity and the appearance of mEH-like activity in the cytosol are novel responses of epoxide metabolizing enzymes, which may be related to the novel cellular responses that follow clofibrate treatment, peroxisome proliferation, hypolipidemia, and nongenotoxic carcinogenesis.

Epoxides are important functional groups on certain arene and alkyl compounds, endogenous and xenobiotic, encountered naturally or after biotransformation. Epoxide moieties may be quite reactive and have been described as the active functional group involved in the covalent binding of several carcinogens and of other toxic compounds to cellular macromolecules (Miller and Miller, 1977; Chacos *et al.*, 1983). In mammalian cells epoxides are generally metabolized by hydrolysis to diols (epoxide ether hydrolysis, EC 3.3.2.3) (Oesch, 1972; Hammock *et al.*, 1980) or by conjugation with glutathione (glutathione *S*-transferases, EC 2.5.1.18) (Jakoby, 1978). Epoxide hydrolysis primarily is associated with two distinct sets of enzymes, a predominantly membranous enzyme, mEH,[4]

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[4] Abbreviations used: mEH, microsomal epoxide hydrolase; cEH, cytosolic epoxide hydrolase; cGST, cytosolic glutathione S-transferase.

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and a predominantly soluble enzyme, cEH. These enzymes can be distinguished by numerous parameters, including substrate selectivity, pH optima, molecular weight, antigenic determinants, and intracellular distribution (Hammock *et al.*, 1980; Guenthner *et al.*, 1981). Glutathione conjugation of epoxides is catalyzed by cGST, a set of isozymes found predominantly in the soluble cell fraction.

Induction of mEH and cGST after treatment with several diverse xenobiotics is well established (see above reviews). It was reported recently from this laboratory that cEH, along with mEH and cGST activity, is increased in mouse liver after treatments with clofibrate (ethyl-α-(*p*-chlorophenoxyisobutyrate)), di-2-ethylhexylphthalate, and 2-ethylhexanol, while being refractive to several other compounds which increased mEH and/or cGST activity (Hammock and Ota, 1983). Waechter *et al.* (1984) have since found that cEH activity is increased in the cytosol from livers of several strains of mice treated with nafenopin, an analog of clofibrate. These four compounds are the only agents now known to increase cEH activity, and all four belong to a set of compounds known as peroxisome proliferators. These compounds all share the capacity to induce hepatic peroxisomes and elicit a hypolipidemic response (Hess *et al.*, 1965; Reddy *et al.*, 1982; Moody and Reddy, 1978). More recently it has become apparent that they also elicit hepatic cancer after long-term treatment, but are not genotoxic in the standard assays (Reddy *et al.*, 1980; Reddy *et al.*, 1982). The proliferative response of peroxisomes is a distinct cellular response to chemicals, and now a unique response of epoxide meabolism to the same compounds has also been identified.

To facilitate studies on the relationship of increased epoxide metabolism to peroxisome proliferation, it is important to establish the basic principles of treatment (i.e., time course and dose response). The metabolism of radiolabeled *cis*- and *trans*-stilbene oxide is a sensitive assay to differentiate among cEH, mEH, and cGST in mice (Gill *et al.*, 1983a; Oesch and Golan, 1980; Hammock *et al.*, 1980). These assays have now been used to study the time course and dose response of the increase in cEH, mEH, and cGST activity in liver from clofibrate-treated mice. The complex subcellular distribution of the three enzymes has been taken into consideration, and the relationship between the increase in epoxide metabolism and peroxisome proliferation is discussed. The increase of cEH activity by peroxisome proliferating–hypolipidemic–carcinogens may provide important information on the physiological role of cEH and the mode of action of these compounds.

METHODS

Materials. Clofibrate was provided by Ayerst Laboratories (New York, N.Y.). *trans*-Stilbene oxide, *cis*-stilbene, *n*-dodecane (99%), and hexanol (98%) were purchased from Aldrich Chemical Company (Milwaukee, Wisc.). *cis*-Stilbene oxide and radiolabeled *cis*- and *trans*-stilbene oxides were prepared as previously described (Hammock and Hasegawa, 1983; Gill *et al.*, 1983a). Radiolabeled benzo[*a*]pyrene-4,5-oxide was provided by F. Oesch (Mainz, West Germany). Bovine serum albumin (fraction V) and glutathione were purchased from Sigma Chemical Company (St. Louis, Mo.). Bio-Rad protein reagent was purchased from Bio-Rad Laboratories (Richmond, Calif.). ACS scintillation fluid was purchased from Amersham (Arlington Heights, Ill.).

Animals, treatment, and preparation of cell fractions. Male and female Swiss–Webster mice (Bantin-Kingman, Fremont, Calif., 25 to 30 and 20 to 30 g, respectively) were housed in steel cages with kiln-dried pine shavings as bedding in an environmentally controlled room (12-hr light cycle, 22.5 to 24.0°C, constant humidity). Feed (Purina Rodent Chow) and tap water were provided *ad libitum*. Clofibrate dissolved in corn oil was mixed into ground chow at concentrations from 0.05 to 2.0% (w/w). Control and clofibrate-withdrawn diets contained similar amounts of corn oil (10 ml/100 g; this results in a total fat content of 15% resulting in a nonisonutrient diet). Fasted mice (used only when indicated) had feed removed 16 hr prior to termination. Mice were killed by cervical dislocation between 9 and 10 AM; livers were removed, perfused with cold 1.15% KCl, placed into buffer, and weighed. Homogenates (20-sec burst with polytron) from individual livers were then used to prepare cell fractions. Conventional crude microsomal and cytosolic fractions were prepared from 10% (of original liver weight, w/v) homogenates in 76 mM sodium phosphate (pH 7.4) as described by Hammock and Ota

(1983), with microsomes subsequently washed in 0.05 M Tris–HCl (pH 7.4), 0.125 M KCl. Alternatively, 10% hepatic homogenates prepared in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4) were separated by differential centrifugation as described by Gill and Hammock (1981) and presented in histogram form as described by de Duve *et al.* (1955). All fractions from this separation and the above preparations were either assayed fresh or stored at −70°C. No difference in activity was noted between fresh and frozen preparations. Centrifugation was performed with Sorvall SS-34 or Beckman 42.1 rotors at 4°C.

Enzyme assays. The standard assay conditions for cEH, mEH, and cGST activity by radiometric partition assay using *trans-* and *cis*-stilbene oxides as substrate have been described by Gill *et al.* (1983a). In brief, cEH activity was assayed as the formation of diol from *trans*-[^{3}H]stilbene oxide at pH 7.4, mEH activity as formation of diol from *cis*-[^{3}H]stilbene oxide at pH 9.0, and cGST activity as formation of glutathione conjugate from *cis*-[^{3}H]stilbene oxide at pH 7.4. The validity of the assays under these conditions was shown by Gill *et al.* (1983a) and Hammock and Hasagawa (1983). The hydrolysis of benzo[*a*]pyrene-4,5-oxide was assayed as described by Jerina *et al.* (1977). All assays were performed in triplicate. Scintillation counting was performed in a LKB 1217 Rackbeta liquid scintillation counter.

Protein assay. Protein was measured by a modification of the method of Bradford (1976) which is suitable for an ELISA reader. Protein or bovine serum albumin (100 μl) was added to a single cuvette, followed by the addition of 400 μl of diluted Bio-Rad reagent, and the mixture was incubated for 10 min at room temperature. Absorbance at 620 nm was then determined with a Gilford EIA manual reader.

Calculations and statistics. The enzyme activities were routinely calculated as specific activity (per milligram of protein), hepatic concentration (per gram of liver), and total relative activity (per liver of 100 g mouse) with specific activities generally presented in the text. The significance of differences between treated and control groups was estimated by Student's t test with $p < 0.05$ accepted as significant.

RESULTS

Control Values

The majority of these experiments was performed on crude microsomal and cytosolic fractions prepared from livers of male and female Swiss–Webster mice. The individual control values for activities of mEH, cEH, and cGST (Fig. 1) from male (n = 52) and female (n = 9) are presented in histogram form. The histograms readily display the variance in values which were encountered over the course of the experiments. Furthermore, sexual differences are apparent. Relative liver weight, microsomal protein (data not shown), and cEH and mEH activities were significantly greater in males, while cGST activity was significantly greater in female mice. The values presented here can be applied to later data presented as percentage of control to calculate the actual values.

Assay for Epoxide Metabolism

The differentiation of epoxide metabolism by cEH, mEH, and cGST is dependent upon substrate specificity, pH optima, and cell fraction used. *cis-* and *trans*-Stilbene oxide metabolism conveniently and sensitively differentiates among cEH, mEH, and cGST in untreated mice (Gill *et al.*, 1983a). By these methods, the three enzyme activities are operationally defined as cEH—hydrolysis of *trans*-stilbene oxide by cytosolic protein at pH 7.4; mEH—hydrolysis of *cis*-stilbene oxide by microsomal protein at pH 9.0; and cGST—conjugation of *cis*-stilbene oxide in the presence of glutathione by cytosolic protein at pH 7.4. The use of single compounds or geometrical isomers of a single compound for the assays enhances studies comparing different enzymes involved in epoxide metabolism. In tissue from livers of control and clofibrate-treated mice, these assays were adequate to detect activity in the major cell fractions of the enzymes (mEH—microsomes; cEH and cGST—cytosol), as well as cell fractions with minor amounts of activity. Formation of product for all three assays was linear with respect to time of incubation and protein added until approximtely 40% of substrate was consumed (data not shown). Activities reported were always obtained from assays within the linear range.

The response of cEH, cGST, and mEH activities in microsomes and cytosol from

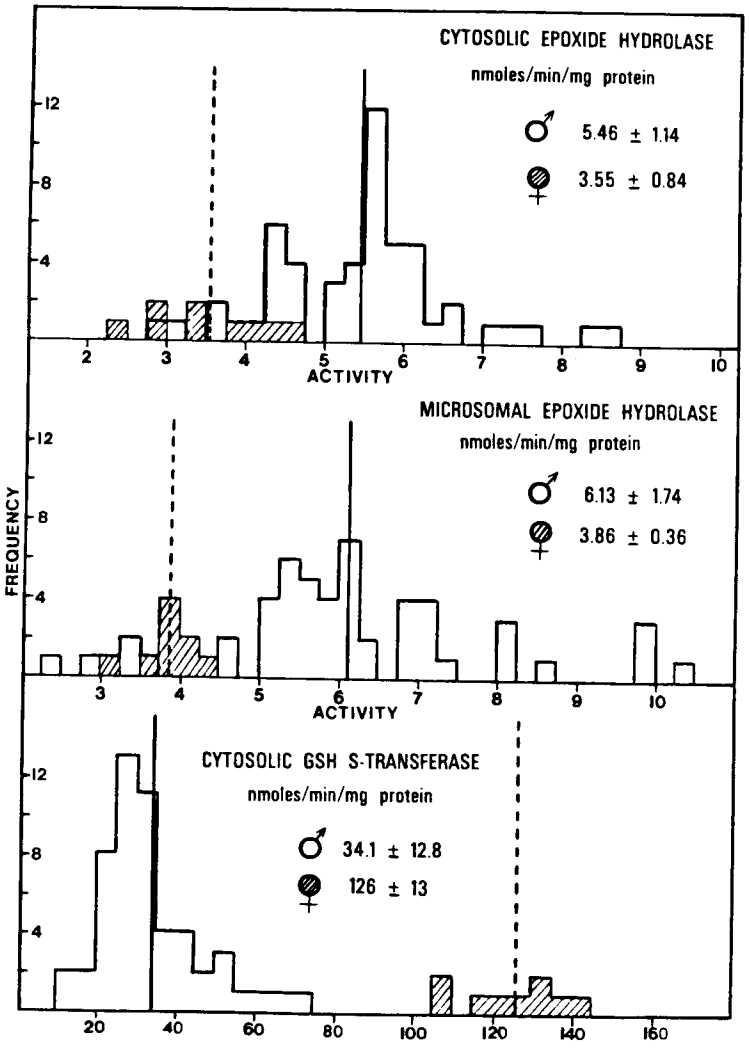

FIG. 1. Control values for cEH, mEH, and cGST activities in male and female mice. Male and female Swiss–Webster mice were used in all experiments. Livers were removed, microsomal and cytosolic fractions prepared, and cEH, mEH, and cGST activities determined as described under Methods. The values for the above parameters were plotted on the abscissa vs numbers of individual male (n = 51, open squares) and female (n = 9, hatched squares) mice on the ordinate in histogram form. $\bar{x} \pm 1$ SD are given and are representative of control values in subsequent figures presented as percentage of control.

mouse liver to treatment for 14 days with clofibrate is summarized in Table 1. The hydrolysis of benzo[*a*]pyrene-4,5-oxide at pH 9.0 also was assayed in both fractions as this substrate is not metabolized detectably by cEH (Hammock and Hasegawa, 1983; Oesch and Golan, 1980; Wang *et al.*, 1982) and may provide a more reliable assessment of increase of mEH-like activity in cytosol than *cis*-stilbene oxide. Activities are expressed per gram of liver to facilitate comparison between the two cell fractions. It is readily apparent that the majority of the activity of cEH (ratio of cytosol to microsomes 250 and 170) and cGST (ratio of cytosol to microsomes 2540 and 1450) is present in the hepatic cytosol

TABLE 1

TSO (pH 7.4), CSO (pH 9.0), AND BPO (pH 9.0) HYDROLYSIS AND CSO CONJUGATION WITH GLUTATHIONE (pH 7.4) IN MICROSOMES AND CYTOSOL FROM CONTROL AND CLOFIBRATE-FED MICE[a]

Sample	Substrate				
	TSO pH 7.4	CSO and GSH pH 7.4	CSO pH 9.0	BPO pH 9.0	BPO/CSO pH 9.0
	nmol/min/g liver				
Control cytosol	246 ± 30	1500 ± 160	44 ± 5 (37)[b]	3.6 ± 2.4	0.08 (0.10)[b]
Clofibrate cytosol	636 ± 71[c]	2910 ± 570[c]	110 ± 10[c] (91)	16.0 ± 0.9[c]	0.14 (0.18)
Control microsomes	1.02 ± 0.25	0.59 ± 0.03	38 ± 6 (38)	28.1 ± 4.4	0.73 (0.73)
Clofibrate microsomes	3.67 ± 0.40[c]	2.00 ± 0.40[c]	87 ± 13[c] (87)	65.2 ± 9.2[c]	0.75 (0.75)

[a] Microsomes and cytosol from livers of mice fed control and 0.5% clofibrate-containing diets for 14 days were prepared and incubated with *trans*-stilbene oxide (TSO), *cis*-stilbene oxide (CSO), ±glutathione (GSH), and benzo[*a*]pyrene-4,5-oxide (BPO) at the specified pH, and the rate of diol or conjugate formation was determined. Values are presented as activity per gram of liver to demonstrate the relative contribution of the two cell compartments to epoxide hydrolysis and conjugation and are $\bar{x} \pm$ SD for three mice.

[b] Values in parentheses were calculated assuming that CSO hydrolysis at pH 9.0 by purified cEH proceeds at a rate of 3% of TSO hydrolysis at pH 7.4. (unpublished data).

[c] Significantly different from the respective control cell fraction, $p < 0.05$.

of control and clofibrate-fed mice, respectively (Table 1). Localization of mEH activity to the microsomal fraction, however, is not so straightforward. Hydrolysis of *cis*-stilbene oxide as nanomoles per minute per gram of liver was actually greater in the cytosol than microsomes from control and clofibrate-fed mice (1.16 and 1.26 ratios, respectively) (Table 1). Preliminary data (unpublished) using purified cEH suggest that *cis*-stilbene oxide (at pH 9.0) is hydrolyzed by cEH at 3% of the rate of *trans*-stilbene oxide (pH 7.4). When the cEH-mediated hydrolysis of *cis*-stilbene oxide is subtracted from *cis*-stilbene oxide hydrolysis in cytosol, the ratios of cytosol to microsomal activity are 0.97 and 1.05 for control and clofibrate-fed mice, respectively (Table 1).

A major contribution to hydrolysis of *cis*-stilbene oxide, apparently not attributable to cEH, was located in the cytosol. The hydrolysis of benzo[*a*]pyrene-4,5-oxide at pH 9.0 was predominantly in the microsomes. The ratios of cytosol to microsome activity was 0.13 and 0.24 for control and clofibrate-fed mice, respectively. These data also demonstrate that the mEH-like activity in the mouse hepatic cytosol has a different ratio of *cis*-stilbene oxide to benzo[*a*]pyrene-4,5-oxide hydrolysis (0.10 and 0.18 for control and clofibrate fed) than the activity in microsomes (0.73 and 0.75 for control and clofibrate fed). The presence of benzo[*a*]pyrene-4,5-oxide hydrolysis in the cytosol suggests that activity other than cEH is located in the cytosol. It is, however, not completely identical to mEH activity in the microsomes.

Dose Response in Male and Female Mice

Sex-dependent differences in the activities of mEH and cGST are well established (Fig. 1) (Oesch, 1972; Hammock *et al.*, 1980; Jakoby, 1978) and sex dependence of the dose response of peroxisome proliferation

and induction of peroxisome-associated enzymes to clofibrate treatment have been described (Svoboda *et al.*, 1969; Reddy and Kumar, 1979). The dose response of cEH, mEH, and cGST activities to clofibrate has therefore been studied in both sexes of mice.

Inclusion of different doses of clofibrate in the diets of male and female mice for 14 days resulted in dose-dependent increases in liver weight and microsomal protein in both sexes and in cytosolic protein in male mice. Inclusion of 2% clofibrate in the diet appeared to be toxic as it resulted in decreases in body weight of male and female mice to 78 and 68% of control, respectively, while no significant change in body weight occurred with lower doses (data not shown). The response of cEH, mEH, and cGST activities was dependent upon dose and sex (Figs. 2A and B). While mEH activity in male and female mice and cGST activity in male mice showed incremental increases with successively higher doses up to 2.0%, the maximal increase in cEH activity occurred at lower doses of 0.5 and 1.0% in male and female mice, respectively. In female mice cGST activity was decreased by doses of clofibrate greater or equal to 0.25% (Fig. 2B). When the difference in activities between control males and females are noted, it is of interest that the maximal alterations of cEH and cGST activities resulted in essentially identical specific activity in both sexes of 14 nmol/min/mg protein for cEH and 88 nmol/min/mg protein for cGST. The ratio of female to male mEH activity also increases from 0.64 in control mice to 0.85 after treatment with the various doses of clofibrate.

Time Course of Increase and Reversion of Activities

The time course of clofibrate-mediated changes and subsequent response to withdrawal of clofibrate from the diet was determined in male mice fed diets containing 0.5% clofibrate (Fig. 3). Liver weight, microsomal protein, and

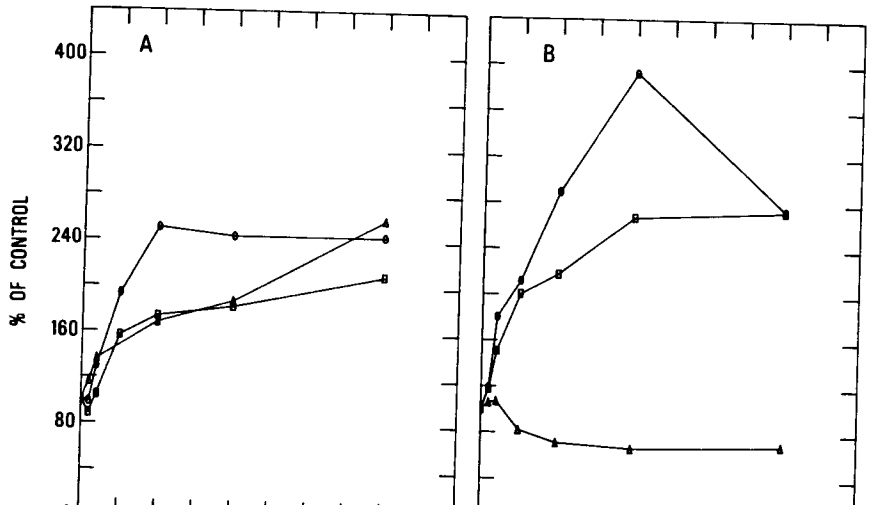

FIG. 2. Dose response to clofibrate in male and female mice. Clofibrate was mixed into diets at the concentrations indicated and fed to male and female mice for 14 days. Livers were removed from (A) male and (B) female mice, cytosolic and microsomal fractions prepared, and cEH (○), mEH (□), and cGST (△) activities determined. Values are the $\bar{x}$ of three to four individual mice expressed as percentage of three to four control mice killed at the same time.

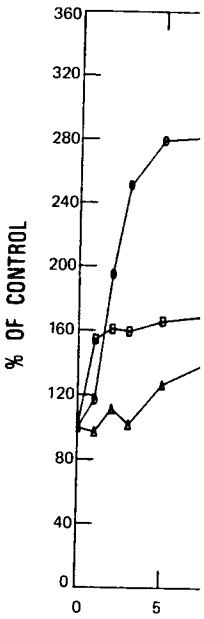

FIG. 3. Time course
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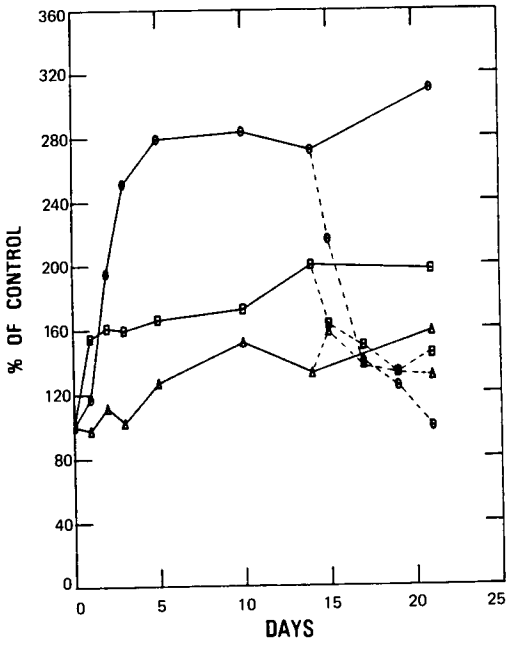

FIG. 3. Time course of response to clofibrate. Male mice were fed diets of 0.5% clofibrate for the times indicated (solid lines), and cEH (○), mEH (□), and cGST (△) activities were determined on the respective cell fractions. In one set of mice the clofibrate diet was replaced with control diet after 14 days of treatment and the effect of withdrawal tested (dashed lines). See legend to Fig. 2 for preparation of tissue and expression of values.

somal protein, and cytosolic protein reached their maximal elevations at 10, 5, and 14 days of treatment, respectively, and returned to control values within 7 days of withdrawal of clofibrate from the diet (data not shown). The maximal increase of cEH activity occurred within 5 days and thereafter remained constant for up to 21 days of treatment. Removal of clofibrate from the diet after 14 days resulted in a rapid decrease in activity, with a return to control values within 7 days after withdrawal. The activity of mEH increased within 1 day to 155% of controls and then further increased with additional treatment to 190% of controls at 14 days of treatment. Within 7 days after withdrawal of clofibrate from the diet, mEH activity was reduced to 120% of control values. cGST activity increased progressively with time on the diet reaching a maximum of 160% of control at 14 to 21 days. Following withdrawal of clofibrate, cGST activity increased initially and then declined toward control values to 125% of controls at 7 days after withdrawal (Fig. 3).

Distribution of Activities between Particulate and Soluble Cell Fractions

The primary localization of activities of cEH and cGST in cytosol and of mEH in the endoplasmic reticulum of hepatic cells of control animals is well established. Numerous reports are available, however, of minor activities of these enzymes in other cell compartments. cEH activity has been described in mitochondrial (Gill and Hammock, 1981), microsomal (Guenthner and Oesch, 1983), and peroxisomal (Waechter *et al.*, 1983) fractions; mEH activity has been described in nuclei, golgi, plasma membrane (Dansette *et al.*, 1979; Stasiecki *et al.*, 1980), and cytosol (Griffin and Noda, 1980; Wang *et al.*, 1982; Gill *et al.*, 1983b), and cGST activity in microsomes (Morgenstern *et al.*, 1980) and mitochondria (Wahllander *et al.*, 1979; Stasiecki *et al.*, 1980). The determination of the exact subcellular localization of all three of these enzymes in control and induced animals is of considerable interest, but beyond the scope of this initial study. For the purposes of this study, we were interested in testing whether the subcellular distribution of the three enzyme activities involved in epoxide metabolism was differentially affected by clofibrate treatment. Homogenates of livers from control and clofibrate-treated mice were separated into five fractions by differential centrifugation. The distribution of cEH, mEH, and cGST activities from control mice was consistent with the primary localizations of the enzymes in cytosol, microsomes, and cytosol, respectively. The polydispercity of the enzymes also is apparent, albeit not well defined, from these studies (Fig. 4). In mice

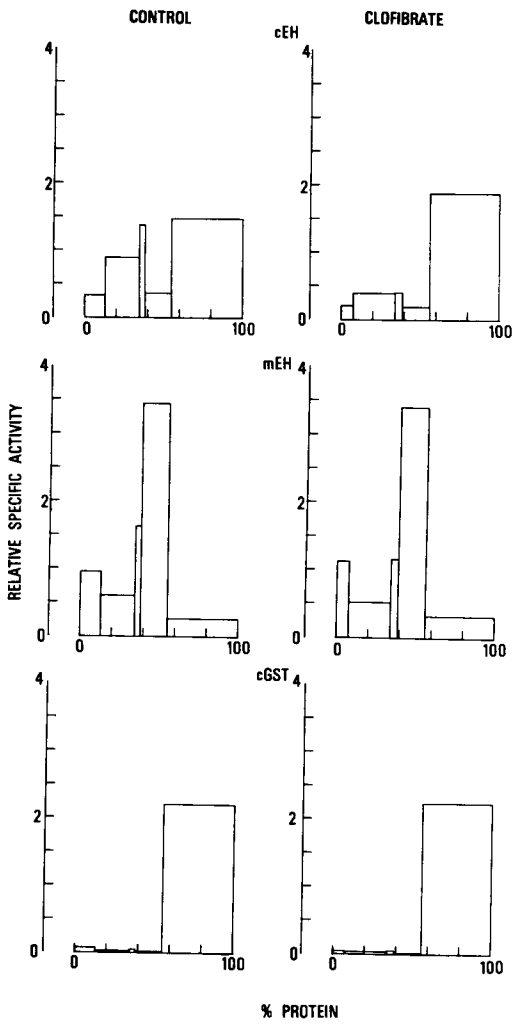

FIG. 4. Effect of clofibrate on the subcellular distribution of cEH, mEH, and cGST. Male mice were fed 0.5% clofibrate for 14 days and fasted overnight. Tissue was fractionated by differential centrifugation and enzymes were assayed as described under Methods. The abscissa represents cumulative percentage protein, and the ordinate, percentage of enzyme activity over percentage of protein (relative specific activity), as suggested by de Duve *et al.* (1955). Values are the $\bar{x}$ of four individual mice.

treated with clofibrate, the distribution of cEH activity was altered noticeably, with decreased relative specific activities in all of the particulate fractions. The distribution of mEH and cGST activities was not as markedly altered, but the relative specific activities of both enzymes in the soluble fraction were increased (Fig. 4).

The differential recovery of the enzymes in soluble vs particulate fractions from control and clofibrate-treated, fasted, or nonfasted mice was examined in greater detail (Table 2). The relative increases in activities of cEH, mEH, and cGST were similar in fasted and nonfasted mice (data not shown). Expression of the recovery in the cytosol as a percentage of total recovery revealed that 42 to 45% of the protein was recovered in this fraction, and this recovery was not altered by clofibrate treatment with or without prior fasting. In a

TABLE 2

RECOVERY OF SOLUBLE PROTEIN, cEH, mEH, AND cGST AFTER DIFFERENTIAL CENTRIFUGATION FROM FASTED AND UNFASTED MICE[a]

Treatment	Protein	cEH	mEH	cGST
	% (Cytosol/sum of fractions)			
Control unfasted	41.5 ± 2.7	67.2 ± 1.3	25.8 ± 1.8	97.4 ± 1.3
Control fasted	44.6 ± 2.0	65.6 ± 2.9	11.4 ± 2.0[b]	97.9 ± 0.3
14-Day clofibrate unfasted	42.0 ± 1.5	84.9 ± 1.1[b]	38.2 ± 2.7[b]	97.9 ± 0.5
14-Day clofibrate fasted	43.9 ± 1.2	82.8 ± 1.5[c]	14.5 ± 3.3[c]	98.0 ± 0.6

[a] Male Swiss–Webster mice were fed diets of 0.5% (w/w) clofibrate or ground chow alone for 14 days. Feed was removed from half the mice in each group 16 hr prior to termination. Livers were fractioned by differential centrifugation and assays were performed as described under Methods. Values are activity or content in cytosol as a percentage of the sum of activity in all fractions and are $\bar{x} \pm$ SD for four mice.

[b] Significantly different from unfasted control, $p < 0.05$.

[c] Significantly different from fasted control, $p < 0.05$.

similar fashion the recovery of cGST activity in the cytosol was consistent (97.4 to 98.0%) regardless of treatment or availability of feed. The recovery of cEH activity in the cytosol was not affected by fasting, but increased from 65 to 68% in control mice to 83 to 85% in clofibrate-treated mice. The recovery of mEH-like activity in the cytosol was lower in the fasted mice, but increased in either situation after clofibrate treatment (Table 2).

DISCUSSION

The increases in activity of epoxide metabolizing enzymes may prove useful in studying the physiological responses associated with peroxisome proliferation including hypolipidemia and carcinogenesis. Conversely, the chemical-mediated induction of enzymes has proven useful in elucidating their biological roles and in providing an enriched source for studies on their physical and chemical characteristics. The recent discovery that cEH activity was increased by four compounds also known to induce hepatic peroxisomes (Hammock and Ota, 1983; Waechter *et al.*, 1984) has now provided a model system to study the increase in cEH activity.

Differential responses in the dose response and time response of increases in cEH, mEH, and cGST activities were seen. cEH activity was maximally increased at a lower dose while mEH and cGST activities (male mice only) were incrementally increased up to the largest dose tested. In addition, the rates of increase with treatment and subsequent reversion of enzyme activity after withdrawal were different. Half-lives for the rate of reversion of increased enzyme activities calculated as suggested by Haining (1971) were 0.70, 4.20, 1.61, 2.35, 2.18, and 2.35 days for cEH, cGST, cytosolic protein, mEH, microsomal protein, and relative liver weight, respectively. The similar rates of reversion for mEH, microsomal protein, and relative liver weight are noteworthy, as are the distinctly different rates of cEH, cGST, and mEH. As these latter two enzyme activities also are increased by numerous other drugs, it is possible that a discrete mechanism of response exists between cEH on one hand and mEH and cGST on the other hand. Lalwani *et al.* (1983) have shown that nafenopin, a potent peroxisome proliferator, binds to cytosolic protein in rats and suggest from this preliminary evidence that peroxisome proliferation is mediated by a cytosolic receptor. One might speculate that cEH, but not mEH and cGST, is also under the control of this putative cytosolic receptor.

The subcellular distribution of cEH, mEH, and cGST activity is characterized by primary localization within either the cytosol or microsomes, as well as minor localization within several other compartments of the cell. Our initial studies with "conventional" cytosolic and microsomal fractions indicated that increase of cEH, mEH, and cGST activity following treatment with clofibrate clearly occurred in the major cell fraction, as well as within a minor cell fraction. Additional studies using differential centrifugation further indicated that the increase of both mEH and cEH activities was associated with a shift of relative specific activity from particulate to soluble fractions. Hydrolysis of *cis*-stilbene oxide and benzo[*a*]pyrene-4,5-oxide occurred in cytosol from both control and clofibrate-treated mice. Based on previous reports (Oesch and Golan, 1980; Guenthner *et al.*, 1981; Wang *et al.*, 1982), cEH has an extremely low activity toward benzo[*a*]pyrene-4,5-oxide. It therefore can be concluded that clofibrate does increase the activity of an "mEH-like" enzyme in cytosol. The difference between microsomes and cytosol in their ratio of hydrolysis of *cis*-stilbene oxide to benzo[*a*]pyrene-4,5-oxide suggests that this enzymatic activity may also be different from mEH. This speculation deserves more rigorous study.

While the predominance of activity characteristic of cEH is indisputably in the cytosol, the present study on the distribution of cEH activity after differential centrifugation of control mouse liver agrees with the previous studies (Gill and Hammock, 1981; Guenthner and Oesch, 1983; Waechter *et al.*, 1983) which clearly indicate a particulate localization of cEH. The high relative specific activity of cEH in the light mitochondrial fraction and the increase in activity of cEH by peroxisome proliferators (Hammock and Ota, 1983; Waechter *et al.*, 1984) presents an intellectually attractive argument for the increase of cEH in peroxisomes. However, the relative specific activity of cEH in the light mitochondria fraction is actually decreased after treatment with the peroxisome proliferator clofibrate. A similar response to peroxisome proliferators is seen with the peroxisomal enzyme catalase which may arise from breakage of peroxisomes (Leighton *et al.*, 1975; Moody and Reddy, 1976) or from true localization of portions of catalase in the cytosol (Jones and Masters, 1978). Potential induction of cEH in peroxisomes may similarly be difficult to resolve.

The cGST activity measured in this study was limited to the conjugation of *cis*-stilbene oxide for comparison with the substrates used for hydrolysis. Measurement with a single substrate would not distinguish the multiple isozymes of cGST in mouse liver (Lee *et al.*, 1981). However, our previously described decrease in conjugation of 1,2-dichloro-4-nitrobenzene in livers of male mice fed clofibrate (Hammock and Ota, 1983) and the sex dependence of the response of *cis*-stilbene oxide conjugation described in this report suggest that the response of mouse liver cGST to clofibrate may be isozyme dependent. No evidence for an isozyme-dependent response of cEH or mEH to clofibrate treatment is evident from these or previous studies.

The increase of cEH activity by clofibrate and other peroxisome proliferators is limited so far to this class of compounds (Hammock and Ota, 1983; Waechter *et al.*, 1984). While the increase of cEH activity does appear to be coupled with the proliferation of peroxisomes, the increases in mEH and cGST activities in this study and following treatment with several other xenobiotics may be coupled with the proliferation of the smooth endoplasmic reticulum and cytochrome *P*-450-dependent mixed-function oxidase (Oesch, 1972) which also occur along with peroxisome proliferation (Moody and Reddy, 1976; Orton and Parker, 1982). The mechanisms by which the activities of the epoxide metabolizing enzymes increase have not been fully elucidated. Whether induction or activation of the enzymes occurs is not even known, but their association with other cellular responses,

peroxisome proliferation and induction of cytochrome *P*-450, may help explain the physiological role of the increase in activity of these epoxide metabolizing enzymes. Since both the cytochrome *P*-450 system and the peroxisomal oxidase–catalase systems are involved in producing active forms of oxygen which could in turn generate epoxides, one may speculate that the coupled increases in activity of mEH, cGST, and cEH arise as a protective mechanism.

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