J. Pharm. Pharmacol. 1993, 45: 458–465
Received June 8, 1992

Effect of Hepatotoxic Doses of Paracetamol and Carbon Tetrachloride on the Serum and Hepatic Carboxylesterase Activity in Mice*

TIEN L. HUANG†, SERGIO A. VILLALOBOS AND BRUCE D. HAMMOCK

Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616, USA

Abstract—The carboxylesterase activity in the serum and liver of untreated Swiss-Webster mice, and in mice administered hepatotoxic doses of either CCl_4 or paracetamol was studied. In addition to *p*-nitrophenyl acetate (*p*-NpAc) and diethylsuccinate, a sensitive, spectrophotometric substrate, methyl β-(1-pentylthio) propiothioate was used to determine the esterase activity. At 24 h after treatment with CCl_4 (1 mL kg^{-1}), the liver esterase activity in the soluble fraction acting on *p*-NpAc was increased 1·7-fold whereas the microsomal esterase activity decreased by one-half. The serum esterase activity increased 2·4- to 3·4-fold depending upon the substrate used. Esterase activity assays of sliced gels from isoelectric focusing (IEF) of serum from mice treated with CCl_4 indicated the presence of at least three additional esterase peaks when compared with serum of control mice. These peaks correlated with esterase bands visualized after staining the IEF gel with 1-naphthyl acetate. Furthermore, these esterase bands matched closely the esterase bands from microsomes of normal mice. The serum esterase activity was analysed at 4, 8, 12 and 24 h after paracetamol (400 mg kg^{-1}) treatment. Serum esterase activity remained unchanged or decreased marginally depending on the treatment time and substrate used. Serum glutamic oxalacetic transaminase levels in CCl_4- and paracetamol-treated mice, however, were significantly elevated compared with control mice. These results suggest that acute liver damage might cause the release of carboxylesterase activity to the soluble intracellular and extracellular compartments, including blood serum, with some but not all toxicants. The results also indicate that the different modes of action of the two chemicals may account for the difference in the serum carboxylesterase activity of the experimental animals.

The carboxylesterases (E.C.3.1.1.1) comprise a family of isozymes present in the microsomes of most mammalian cells, with the highest concentration occurring in the liver. They are involved in the metabolism of ester, thioester, or amide xenobiotics and thus play an important part in the detoxification system of the body (Heymann 1980; Leinweber 1987; Satoh 1987; Okuda 1991). The liver carboxylesterases are localized on the luminal surface of the endoplasmic reticulum membrane, probably by hydrophobic binding to phospholipids (Akao & Omura 1972; Harano et al 1988). These enzymes can be easily solubilized in-vitro by detergents (Ljungquist & Augustinsson 1971; Akao & Omura 1972) or by lipid peroxidation (Talcott et al 1980) and remain active after liberation from the membrane.

It has been reported that the serum carboxylesterase activity shows a remarkable increase in patients with liver damage resulting from overdose of paracetamol, myocardial infarction with secondary liver necrosis, and hepatic cirrhosis (Talcott et al 1982; Hammock et al 1984). An excellent correlation between esterase activity and serum glutamic oxalacetic transaminase (SGOT) levels was reported in these studies. These observations suggested that the relatively high carboxylesterase activity in the liver is released into the serum following liver injury. The present study was undertaken, in part, to determine if the mouse is an adequate model for such studies.

In the present investigation, the carboxylesterase activity in the sera of mice treated with hepatotoxic levels of paracetamol or CCl_4 was measured. The effect of CCl_4 in solubilizing the membrane bound carboxylesterases into the soluble fraction as a result of hepatic membrane damage was also studied. An additional purpose of this study was to evaluate the utility of a new substrate which is turned over more rapidly by human and murine esterases than currently used substrates (Huang et al 1992). A long-term goal is to evaluate the potential value to medical and veterinary diagnosis of including esterase assays as one of a battery of serum tests for hepatic dysfunction.

Materials and Methods

Materials

Paracetamol, CCl_4, *p*-nitrophenol, *p*-nitrophenyl acetate (*p*-NpAc), diethylsuccinate (DES), glycerol and fast blue RR salt were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Alcohol dehydrogenase, *p*-iodonitrophenyltetrazolium violet (INT), NAD, NAD diaphorase, bovine serum albumin (fraction V), 1-naphthyl acetate and transaminase diagnostic kit No. 505-OP were purchased from Sigma Chemical Co. (St Louis, MO, USA). BCA protein reagent was purchased from Pierce Chemical Co. (Rockford, IL, USA). Methyl β-(1-pentylthio) propiothioate (MBPTP) was synthesized as described by Huang et al (1992).

Animals and treatments

Male Swiss-Webster mice, 25–30 g, were purchased from

* Part of this work was presented at the 31st annual meeting of the Society of Toxicology, held in Seattle, Washington, February 23–27, 1992.

† Present address: College of Pharmacy, Xavier University of Louisiana, New Orleans, LA 70125, USA.

Correspondence: B. D. Hammock, Department of Entomology, University of California, Davis, CA 95616, USA.

Table 1. Serum carboxylesterase and SGOT activity in mice after administration of paracetamol[a].

Treatment	Number of mice	Time of killing (h)	Carboxylesterase activity[b] (nmol min mL^{-1})			SGOT[b] (int. units L^{-1})
			p-NpAc	DES	MBPTP	
Control	7	24	246±32	27·0±1·6	449±63	45·8±12·8
Paracetamol (300 mg kg^{-1})	7	24	231±9	26·2±1·9	443±42	161±111[c]
Control	5	4	178±21	18·2±1·9	452±59	54±11
	5	8	158±11	18·5±1·2	541±29[d]	58±8·6
Paracetamol (400 mg kg^{-1})[e]	5	4	157±20	13·6±2·9[c]	439±93	4083±236[c]
	3	8	143±13	13·9±3·7[c]	568±58	3350±150[c]
	1	24	139	16·8	473	3500

[a] Mice were treated intraperitoneally with either saline (control) or paracetamol, and serum carboxylesterase and SGOT levels were determined at different time intervals after dosage, as described in Materials and Methods. Paracetamol was administered in saline. [b] Values are the mean±s.d. [c] Significantly different from the respective control, $P<0·05$. [d] Significantly different from 4 h control, $P<0·05$. [e] The 400 mg kg^{-1} dose resulted in 60, 100 and 80% mortality for the 8, 12 and 24 h treated animals, respectively. The data reported for these groups are for the surviving animals.

Bantin-Kingman (Fremont, CA, USA). The mice were housed in steel cages with kiln-dried pine shavings as bedding in a constant temperature (23±1·5°C) and humidity environment on a 12 h light-dark cycle. Food (Purina Rodent Chow) and water were freely available. Animals were acclimatized for one week before the experiment was begun.

Paracetamol at different doses (300 and 400 mg kg^{-1}) was injected intraperitoneally into mice as a near-saturated solution in 0·9% NaCl (saline) (20 μL g^{-1}). Saline was administered to control mice. The dosing was begun at 0900 h and the animals were killed at different times (Table 1). Carbon tetrachloride in corn oil was injected intraperitoneally into mice at a dose of 1 mL kg^{-1}. Control mice were treated with corn oil only. The animals were killed 24 h after dosage.

Enzyme preparation

Mice were killed by exsanguination under CO_2 anaesthesia. Blood samples were collected from the heart (by cardiac puncture) and from the inferior vena cava. The blood was allowed to clot at 4°C and then centrifuged at 2500 rev min^{-1} (1000 *g*) for 30 min at 4°C. Serum was collected and stored frozen at −70°C until analysis.

Liver microsomal and cytosolic fractions of control and CCl_4-treated mice were isolated by differential centrifugation. After the blood had been collected, the livers were perfused with cold 1·15% KCl, removed, rinsed in 0·25 M sucrose-0·1 M Tris-HCl buffer (pH 7·5), and weighed. Livers were minced and homogenized for 20 s using a Polytron on setting 6. The liver homogenates (20% of liver weight, w/v) were centrifuged at 10000 *g* for 15 min, and the supernatant fractions were centrifuged at 100000 *g* for 60 min. The cytosol was collected after drawing off the fatty layer. The pellet was washed with 0·25 M sucrose–0·05 M Tris-HCl buffer (pH 7·5) and resuspended in the same buffer. The microsomes and cytosols were stored at −70°C until used for enzyme assay.

Enzyme assays

The initial rates of hydrolysis of DES, *p*-NpAc and MBPTP by mouse serum and liver carboxylesterases were monitored spectrophotometrically using 96-well microtitre plates (Dynatech Laboratories, Inc., VA, USA) with a V_{max} plate reader (Molecular Devices, Palo Alto, CA, USA). The enzyme assays were carried out under conditions where the initial hydrolysis rates were linear with time and protein concentration. All assays were carried out at 23°C. Independent studies demonstrated that the plate reader gave rates very similar to those observed with a Varian Cary spectrophotometer.

Serum carboxylesterase activity on DES was assayed according to the method of Talcott (1979). The hydrolysis of DES was coupled to the reduction of a tetrazolium dye (INT) by alcohol dehydrogenase and NADH diaphorase. The enzyme activity, which is equivalent to the reduction of INT, was calculated from the linear portion of the change in absorption at 490 nm using an extinction coefficient of 13·8 mM^{-1} cm^{-1}. The assays were monitored for 5 min in wells containing 300 μL of incubation mixture. The mixture contained 50 μL serum (1:10 final dilution in 0·1 M Tris-HCl buffer, pH 7·5 containing 10% glycerol) and 250 μL reagent mixture in 0·1 M Tris-HCl buffer (pH 7·5). The reaction was started by injecting 2 μL DES in acetone (5×10^{-4} M final concentration) into a well using a Hamilton repeating dispenser and stirring the well contents with the syringe needle. As controls, reagent and enzyme blanks were run with the reaction mixture without serum and substrate, respectively.

The hydrolysis of *p*-NpAc was assayed as described by Ljungquist & Augustinsson (1971) and Ashour et al (1987). The liberation of *p*-nitrophenol was monitored for 2 min at 405 nm. The incubation mixture contained 20 μL serum (1:20 final dilution) in 300 μL of 0·1 M Tris-HCl buffer (pH 7·5). The reaction was initiated by injecting 2 μL of an acetone solution of the substrate yielding 5×10^{-4} M final concentration. Blanks containing no enzyme were run and corrections were made for spontaneous substrate hydrolysis. For assaying the esterase activity in liver cytosolic and microsomal fractions of control and CCl_4-treated mice, the incubation mixture contained 50 μL enzyme solution in 300 μL of 0·1 M Tris-HCl buffer (pH 7·5). The remaining assay procedures are the same as described above for the serum esterase assay.

Serum carboxylesterase activity on MBPTP was assayed by a modification of the method of Ellman et al (1961). In a typical assay, 278 μL of 0·015% DTNB in 0·1 M Tris-HCl

Table 2. Serum carboxylesterase and SGOT activity in mice after administration of CCl_4.[a]

	Number of mice (n)	Time of killing (h)	Carboxylesterase activity[b] (nmol min mL^{-1})			SGOT[b] (int. units L^{-1})
			p-NpAc	DES	MBPTP	
Control	7	24	233 ± 18	22·4 ± 1·4	371 ± 50	21·0 ± 7·6
CCl_4 (1 mL kg^{-1})	6	24	713 ± 108[c]	76·5 ± 2·7[c]	901 ± 174[c]	4700 ± 630[c]

[a] Mice were treated intraperitoneally with either corn oil (control) or CCl_4, and serum carboxylesterase and SGOT levels were determined 24 h after dosage as described in Materials and Methods. CCl_4 was administered in corn oil. [b] Values are the mean ± s.d. [c] Significantly different from the respective control, $P < 0·05$.

buffer (pH 7·5), and 20 μL serum (1:20 final dilution) were added to individual wells. The reaction was initiated by the addition of 2 μL MBPTP (in ethanol) to give a final concentration of 2×10^{-4} M. Reagent blanks containing no enzyme were used as controls. The esterase activity was measured by following the increase of yellow colour (anion of 5-thio-2-nitrobenzoic acid) at 405 nm produced from the anion of methanethiol when it reacts with DTNB for 2 min.

SGOT was analysed colorimetrically using the Sigma assay kit No. 505-OP. One int. unit of SGOT is the amount of enzyme that will convert 1 μmol aspartate min^{-1} at pH 7·5 and 37°C.

Electrophoresis and esterase staining

Isoelectric focusing (IEF) was performed on an LKB 2117 multiphor II electrophoresis unit using LKB Ampholine PAGplates. The narrow range precast, 11·0 cm gel (pH 4·0–6·5) was 5% polyacrylamide and 2·2% ampholine. Gels were cut in half and used in these experiments. The gels were prefocused for 30 min at 4°C and 12·5 W without samples. Thirty microlitres of each serum and liver sample was applied on filter wicks in separate lanes excluding 1 cm from the cathode and anode ends. Electrofocusing was conducted for 2·0 h at a constant power of 12·5 W. The gel was then sliced into eighteen 5 mm pieces and eluted overnight at 4°C in 0·5 mL of 0·1 M Tris-HCl buffer (pH 7·5) for measurement of enzyme activity or in 0·5 mL of 20 mM KCl solution for measurement of pH (Figs 1, 2). Each sample was analysed on at least two separate lanes with application in different gel regions to avoid application artifacts. For the IEF experiments, the serum and liver samples were pooled from at least six mice. Serum samples from the 8 h, paracetamol (400 mg kg^{-1})-treated animals were pooled from the surviving three mice.

IEF gels were stained for esterase activity using 1-naphthyl acetate. Following IEF, the gel was preincubated in 0·05 M Tris-HCl buffer (pH 7·5), for 15 min at 23°C and then transferred to the staining solution (100 mL 0·05 M Tris-HCl buffer, pH 7·5, 100 mg of fast blue RR salt, and 4 mL of 1% 1-naphthyl acetate in acetone) for 30 min at 23°C, followed by storage in 5% acetic acid. For gels stained with 1-naphthyl acetate (Fig. 3), the amount of serum (lanes 1, 2, 3), microsomal (lanes 4 and 5), and cytosolic (lanes 6 and 7) protein samples applied were 391, 19·1 and 89·7 μg, respectively.

Protein determination

Protein concentrations were determined using the standard protocol version of the Pierce BCA assay. Bovine serum albumin was used as a protein standard.

Statistical analysis

The esterase activities were calculated as means and standard deviations. Levels of statistical significance were determined using the unpaired Student's *t*-test, with $P < 0·05$ considered significant.

Results

Effect of paracetamol on serum carboxylesterase and glutamic oxalacetic transaminase activity

At 24 h following administration of paracetamol (300 mg kg^{-1}, i.p.) to Swiss-Webster mice, the total serum carboxylesterase activity toward all three substrates was unchanged relative to controls (Table 1). To produce severe hepatic necrosis, the dosage of paracetamol was increased to 400 mg kg^{-1}, and the carboxylesterase activity was measured at different times. This dosage resulted in 60, 100, and 80% mortality for the 8, 12 and 24 h treated animals, respectively. A significant variation in esterase activity measured with MBPTP was observed between the 4 and 8 h control mice. However, no significant difference in esterase activity was observed between the 4 and 8 h control mice with *p*-NpAc and DES as substrates. At 4 and 8 h following treatment, the esterase activity with DES decreased by about 25% when compared with the 4 and 8 h saline-treated control mice, respectively. However, no change in esterase activity with *p*-NpAc or MBPTP was observed with the 4 and 8 h paracetamol-treated mice when compared with the control

Table 3. Effect of CCl_4 on hepatic microsomal and cytosolic carboxylesterase activity in mice[a].

Fraction	Number of mice	*p*-NpAc carboxylesterase activity[b] Specific activity (units (mg protein)$^{-1}$)	Total activity (units)
Microsomal			
Control	6	668 ± 99	6·3 ± 1·7
CCl_4-treated	6	296 ± 49[c]	1·2 ± 0·3[c]
Cytosol			
Control	6	59 ± 5	0·79 ± 0·08
CCl_4-treated	6	100 ± 8[c]	0·87 ± 0·13

[a] Mice were treated intraperitoneally with CCl_4 (1 mL kg^{-1}) 24 h before being killed. Control groups were treated with corn oil. Microsomal and cytosolic fractions were isolated from mice livers as described in Materials and Methods. 1 unit activity = 1 nmol *p*-NpAc hydrolysed min^{-1} at 23°C. [b] Values are the mean ± s.d. [c] Significantly different from the respective control, $P < 0·05$.

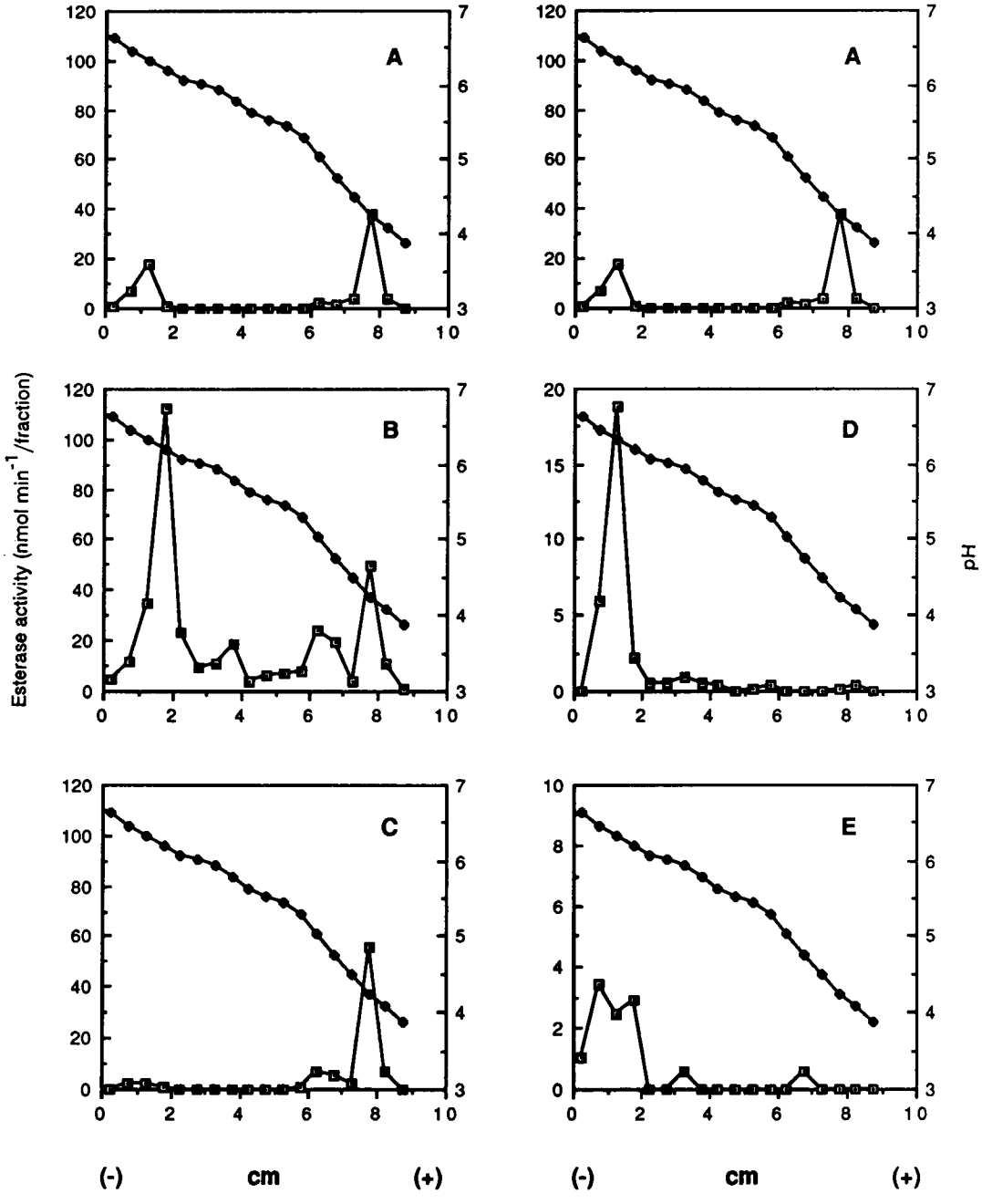

FIG. 1. Isoelectric focusing of serum and liver esterases monitored with *p*-nitrophenyl acetate. Electrophoretic patterns of serum esterase activities in normal mice (A), in mice treated 24 h earlier with CCl_4 (1 mL kg^{-1}) (B), and in mice 8 h after treatment with paracetamol (400 mg kg^{-1}) (C). Electrophoretic pattern of liver microsomal (D) or cytosolic (E) esterase activity in normal mice. Electrofocusing was performed on a horizontal slab gel with a pH 4·0–6·5 gradient for 2 h at 4°C. Following electrofocusing, each lane was sliced into 5 mm segments and eluted overnight with Tris buffer for enzyme activity (□) or 20 mM KCl solution for pH (●) determination. The electrophoretic patterns of serum and liver carboxylesterase activities shown in this figure and in Fig. 2 were repeatable in two separate experiments. A wide-range pH gradient gel (pH 3·5–9·5) was run and no additional peaks of esterase activities were observed.

mice. The SGOT levels were significantly elevated for the paracetamol-treated mice especially at the 400 mg kg^{-1} dose, indicating considerable hepatotoxicity.

Effect of CCl_4 on serum and liver carboxylesterase and glutamic oxalacetic transaminase activity

At 24 h after CCl_4 (1 mL kg^{-1})-treatment, the serum carboxylesterase activity measured with *p*-NpAc, DES, and MBTA increased by 206, 241 and 143%, respectively (Table 2). The SGOT levels were also elevated significantly. A decrease of 56% and an increase of 69% in specific *p*-NpAc carboxylesterase activities was observed in the liver microsomal and cytosolic fractions, respectively of CCl_4-treated mice (Table 3). As a percentage of the total *p*-NpAc activity, the soluble fraction of liver accounted for 11% in control mice and 42% in CCl_4-treated mice.

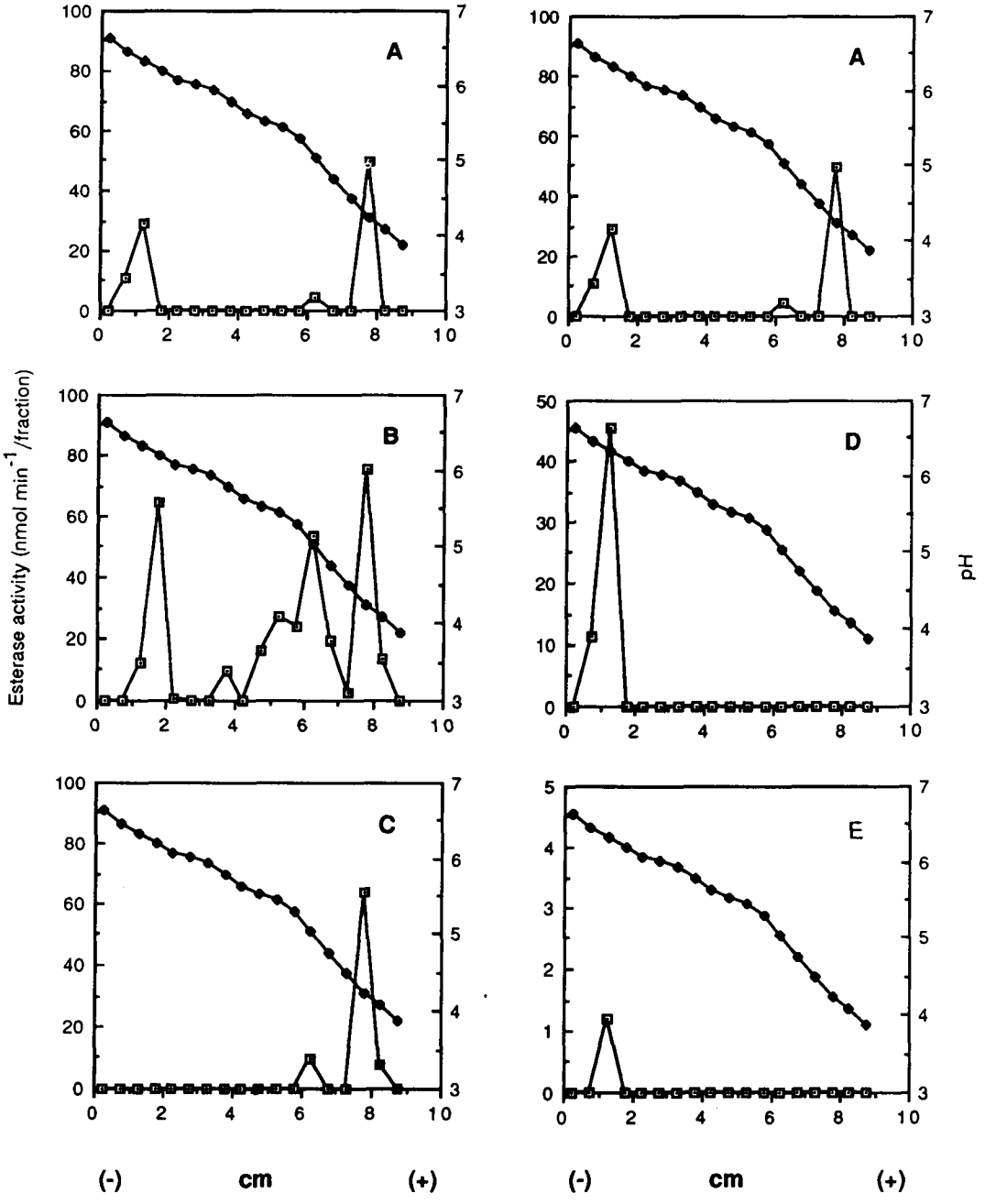

FIG. 2. Isoelectric focusing of serum and liver esterases acting on methyl β-(1-pentylthio) propiothioate. Electrophoretic patterns of serum esterase activities in normal mice (A), in mice treated 24 h earlier with CCl_4 (1 mL kg^{-1}) (B), and in mice 8 h after treatment with paracetamol (400 mg kg^{-1}) (C). Electrophoretic pattern of liver microsomal (D) or cytosolic (E) esterase activity in normal mice. Esterase activities (□) and pH (●) profile were generated by assaying the same samples used for Fig. 1.

Electrophoretic patterns of serum and liver carboxylesterase activities

Carboxylesterase activities with *p*-NpAc and MBPTP in the serum, and liver microsomal and cytosolic fractions were analysed by high resolution IEF. Although the liver microsomes in mammals have been shown to contain multiple forms of carboxylesterases (Mentlein et al 1980; Hosokawa et al 1990), only one peak of activity at an isoelectric point (pI) of 6·3 was detected (Figs 1D, 2D). This peak could represent the major form whereas the other carboxylesterase isozymes may be in low quantities and were not detectable. Alternatively, because of the low recovery (25%) of liver microsomal esterase activity in normal mice, a major fraction of the activity may be unstable during the IEF experiments or in the elution buffer which was incubated overnight. However, the presence of several carboxylesterase isozymes in liver microsomal and cytosolic fractions was visually detected by staining the gel with 1-naphthyl acetate (Fig. 3). The predominant esterase isozyme focused at a higher pI (6·3) and was relatively more abundant than the other

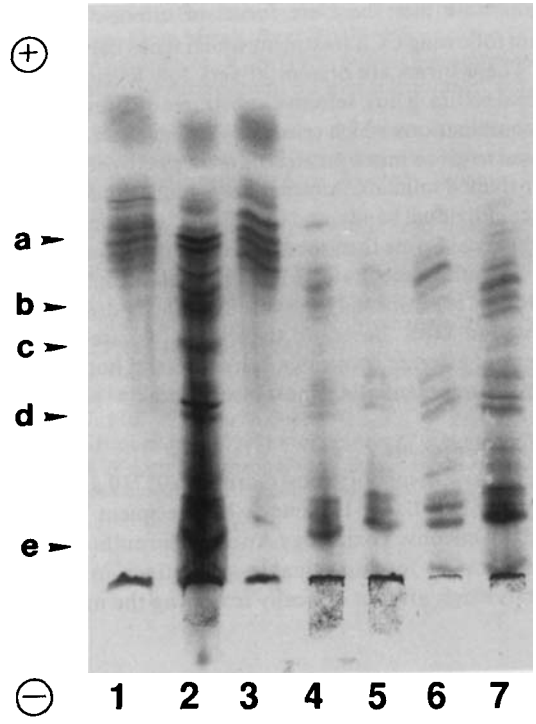

FIG. 3. Isoelectric focusing with narrow-range pH gradient gel (pH 4·0–6·5) of serum and liver esterases stained with 1-naphthyl acetate. Conditions of this experiment are as described for Figs 1, 2. Serum esterase activities in normal mice (lane 1), in mice treated with CCl_4 (1 mL kg^{-1}) (lane 2), and in mice 8 h after treatment with paracetamol (400 mg kg^{-1}) (lane 3) are shown. Liver esterase activities in microsomes of normal mice (lane 4), in microsomes of mice treated with CCl_4 (1 mL kg^{-1}) (lane 5), in cytosol of normal mice (lane 6), and in cytosol of mice treated with CCl_4 (1 mL kg^{-1}) (lane 7) are shown. The amount of serum (lanes 1, 2, and 3), microsomal (lanes 4 and 5), and cytosolic (lanes 6 and 7) proteins applied on the gel were 391, 19·1 and 89·7 μg, respectively. The arrowheads and the corresponding letters indicate the positions of the different esterase isozymes in lane 2. Isozymes with pI values of 4·2, 5·0, 5·5, 5·8, and 6·3 are represented by a, b, c, d, and e, respectively.

isozymes. The carboxylesterases of the cytosolic fraction of liver were very similar to those of the microsomal fraction. The cytosolic esterase activity is usually thought to be the result of autolysis that readily solubilizes the membrane-bound carboxylesterases (Heymann 1980). High esterase activities were observed in the cytosol of mice treated with CCl_4 when compared with control mice and this was evident by the relatively intense esterase bands in the IEF gel stained with 1-naphthyl acetate (Fig. 3, lane 7).

The serum of normal mice showed two peaks of activity at pI values of 4·2 and 6·3 (Figs 1A, 2A). Administration of hepatotoxic doses of CCl_4 (1 mL kg^{-1}) caused a decrease of carboxylesterase activity in the microsomal fraction, and an increase of activity in the cytosolic fraction (Table 3). The peaks of serum carboxylesterase activity (Figs 1B, 2B) were elevated markedly by CCl_4 treatment. Several additional esterases acting on MBPTP were observed at pI values 5·0, 5·5 and 5·8. These esterases correlated with the bands detected after staining with 1-naphthyl acetate (Fig. 3, lane 2). The esterase bands of interest and the corresponding isoelectric points are shown in Fig. 3, lane 2. The serum esterase pattern 8 h after treatment with paracetamol (400 mg kg^{-1}) showed a significant decrease in the esterase peak at pI 6·3 whereas a slight increase in the peak at pI 4·2 was observed (Figs 1C, 2C). The serum esterase bands of normal and paracetamol-treated mice were similar (Fig. 3, lanes 1, 3), although the serum esterase activity at pI 6·3 detected in normal mice was not observed in the 1-naphthyl acetate-stained gel (Fig. 3, lane 1).

Discussion

There are only a few published studies which have demonstrated an association between high serum carboxylesterase levels and acute liver injury (Junge 1978; Junge & Malyusz 1980; Talcott et al 1982; Hammock et al 1984). An immunological technique detected high concentration of carboxylesterases in the sera of patients with necrotizing liver diseases (Junge 1978). Hammock et al (1984) reported that the serum carboxylesterase activity with DES was significantly elevated in patients with liver damage resulting from overdose of paracetamol, myocardial infarction with secondary liver necrosis, and hepatic cirrhosis. The serum carboxylesterase activity with malathion also was found to be elevated in a patient with a paracetamol overdose (Talcott et al 1982). A positive correlation between carboxylesterase activity and SGOT activity was found. The studies suggested that liver damage and carboxylesterase solubilization were related and that serum carboxylesterases may serve as indicators of liver damage.

In the present study, acute treatment with CCl_4 (1 mL kg^{-1}) and paracetamol at doses of 300 and 400 mg kg^{-1} caused significant liver injury in mice. The hepatotoxicity of these chemicals was indicated by the significant increase in SGOT levels.

Following CCl_4 administration, carboxylesterase activity with *p*-NpAc was increased significantly in the soluble fraction of liver whereas the esterase activity in the microsomal fraction decreased. The serum carboxylesterase activity with all three substrates used in the study was increased markedly. A sensitive spectrophotometric substrate, methyl β-(1-pentylthio) propiothioate was used in addition to *p*-NpAc and DES to monitor esterase activity. The decrease of liver microsomal carboxylesterases was paralleled by the marked increase of serum esterase peaks with pI values of 6·3 and 4·2 and the appearance of at least three additional esterase peaks with pI values of 5·0, 5·5 and 5·8, as determined from high resolution isoelectric focusing experiments. The esterase bands observed in the gel stained with 1-naphthyl acetate (Fig. 3, lane 2) strongly correspond to the esterase peaks detected in sliced gels. Furthermore, the esterase bands from the serum of mice treated with CCl_4 closely match the esterase bands from the microsomes of normal mice. Based on these observations, it is reasonable to assume that the serum esterase peaks may represent the different carboxylesterase isozymes of microsomal origin. Thus, these results suggest that acute liver damage caused by CCl_4 in mice causes the release of carboxylesterase activity to the soluble intracellular and extracellular compartments including blood serum. Previous workers (Talcott et al 1980) have suggested that these released microsomal esterases also account for the esterase activity observed in the serum

following liver damage. Our results are consistent with this hypothesis.

On the other hand and in contrast to earlier published studies (Talcott et al 1982; Hammock et al 1984) which reported an association between high serum carboxylesterase levels and paracetamol-induced acute liver injury in man, the results in mice obtained in this study did not support such an observation. The serum carboxylesterase activity in mice was unchanged at a dose of 300 mg kg^{-1}. When the dose was increased to 400 mg kg^{-1}, the serum carboxylesterase activity was either unchanged or decreased marginally. The electrophoretic pattern of serum carboxylesterase from mice treated with paracetamol exhibited a significant decrease in esterase activity, particularly at the peak with pI 6·3. Analysis of the serum esterase bands in the IEF gel stained with 1-naphthyl acetate indicated very similar patterns in normal and paracetamol-treated mice.

It is possible that the different mode of action of these two chemicals might account for the difference in carboxylesterase activity and electrophoretic patterns observed in the serum of the experimental animals. The mechanism of hepatotoxicity of paracetamol is thought to be related to the generation of a reactive electrophilic intermediate, *N*-acetyl-*p*-benzoquinonimine (NAPQI) by the cytochrome P450 mono-oxygenase system (Black 1984; Potter & Hinson 1986, 1987; Nelson & Pearson 1990). NAPQI is efficiently detoxified by conjugation with glutathione at therapeutic doses. However, when hepatic glutathione is depleted by toxic doses, NAPQI binds to protein sulphydryl groups leading to hepatic necrosis. It is possible that reactive metabolites of paracetamol may bind covalently to specific liver esterases, thereby inhibiting the activity of these enzymes. The covalent modification of a microsomal carboxylesterase by the reactive trifluoroacetyl halide metabolite of halothane from liver microsomes of halothane-treated rats has been demonstrated (Satoh et al 1989). A similar mechanism may be occurring with the reactive metabolites of paracetamol. However, unlike in man, the lack of correlation between high serum esterase levels and liver damage in mice may also suggest a species-specific effect by paracetamol. Although several mechanisms of hepatotoxicity have been postulated for CCl_4, the most widely accepted hypothesis is the reductive cleavage of CCl_4 by cytochrome P450 to form free radicals leading to lipid peroxidation of polyunsaturated fatty acids in membranes (Kalf et al 1987). CCl_4-induced lipid peroxidation may account for the release of microsomal carboxylesterase into the extracellular compartments, including blood serum. Further mechanistic studies are needed to clarify the results obtained with paracetamol and CCl_4.

The increase in absolute activity of ester hydrolysis in the serum is much smaller than the increase in SGOT. Also, the increase in esterase activity relative to background is lower than for SGOT suggesting that in the murine systems, the esterase isozymes are not as sensitive as serum indicators of liver necrosis as is SGOT using the substrates reported here. However, there are numerous esterase isozymes expressed differentially in different tissues. In addition, many esterases are simple to monitor. The differential effects on serum esterase levels of CCl_4 and paracetamol suggest that with the proper substrates, esterase assays may provide diagnostic data in addition to SGOT. The IEF data in Figs 1, 2, and 3 demonstrate that there are forms of esterase seen in the serum following CCl_4 treatment which focus between 5·0 and 5·8. These forms are present at very low levels, if at all, in normal serum. Thus, selective substrates or substrate/inhibitor combinations which selectively detect these forms could be used to give a much greater difference between control and CCl_4-treated animals. Alternatively, stains could be used to detect individual bands, and the relative activities of different biochemical forms then used as indicators of tissue damage. The thioether substrate, MBPTP, yields higher rates of hydrolysis in serum and lower background hydrolysis than *p*-NpAc and DES. Based on these data, we are screening a battery of esterase substrates in murine and human systems to optimize potential diagnostic substrates.

Acknowledgements

This work was supported by Grants ES02710 and ES04699 from NIEHS. B. D. Hammock is a recipient of the Burroughs Wellcome Toxicology Award. The authors thank Dr Alan Buckpitt for his valuable suggestions in the experimental design and for critically reviewing the manuscript.

References

Ashour, M.-B., Gee, S. J., Hammock, B. D. (1987) Use of a 96-well microplate reader for measuring routine enzyme activities. Anal. Biochem. 166: 353–360

Akao, T., Omura, T. (1972) Acetanilide-hydrolyzing esterase of rat liver microsomes I. Solubilization, purification, and intramicrosomal localization. J. Biochem. 72: 1245–1256

Black, M. (1984) Acetaminophen hepatotoxicity. Ann. Rev. Med. 35: 577–593

Ellman, G. L., Courtney, K. D., Andres, V., Featherstone, R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88–95

Hammock, B. D., Loury, D. N., Moody, D. E., Ruebner, B., Baselt, R., Milam, K. M., Volberding, P., Ketterman, A., Talcott, R. (1984) A methodology for the analysis of the preneoplastic antigen. Carcinogenesis 5: 1467–1473

Harano, T., Miyata, T., Lee, S., Aoyagi, H., Omura, T. (1988) Biosynthesis and localization of rat liver microsomal carboxylesterase E1. J. Biochem. 103: 149–155

Heymann, E. (1980) Carboxylesterases and amidases. In: Jakoby, W. B., Bend, R. R., Caldwell, J. (eds) Enzymatic Basis of Detoxication. Academic Press, New York, pp 291–323

Hosokawa, M., Maki, T., Satoh, T. (1990) Characterization of molecular species of liver microsomal carboxylesterases of several animal species and humans. Arch. Biochem. Biophys. 277: 219–227

Huang, T. L., Uematsu, T., Li, Q. X., Borhan, B., Hammock, B. D. (1992) Solubilization and structure-activity relationships for substrates and inhibitors of mammalian liver microsomal carboxylesterases. The Toxicologist 12: Abstract 1651; 31st Annual Meeting of the Society of Toxicology, Seattle, WA, February 23–27, 1992

Junge, W. (1978) Human microsomal carboxylesterase (EC 3.1.1.1). Distribution in several tissues and some preliminary observations on its appearance in serum. In: Goldberg, D. M., Wilkinson, J. H. (eds) Enzymes in Health and Disease. Karger, Basel, pp 54–58

Junge, W., Malyusz, M. (1980) Microsomal carboxylesterase (EC 3.1.1.1). A serum indicator of endoplasmic reticulum injury in hypoxemic liver disease. In: Goldberg, D. M., Werner, M. (eds) Progress in Clinical Enzymology. Masson Publishing, New York, pp 187–190

Kalf, G. F., Post, G. B., Snyder, R. (1987) Solvent toxicology: recent advances in the toxicology of benzene, the glycol ethers, and carbon tetrachloride. Annu. Rev. Pharmacol. Toxicol. 27: 399–427

Leinweber, F.-J. (1987) Possible physiological roles of carboxylic ester hydrolases. Drug Metab. Rev. 18: 379–439

Ljungquist, A., Augustinsson, K.-B. (1971) Purification and properties of two carboxylesterases from rat liver microsomes. Eur. J. Biochem. 23: 303–313

Mentlein, R., Heiland, S., Heymann, E. (1980) Simultaneous purification and comparative characterization of six serine hydrolases from rat liver microsomes. Arch. Biochem. Biophys. 200: 547–559

Nelson, S. D., Pearson, D. (1990) Covalent and noncovalent interactions in acute lethal cell injury caused by chemicals. Annu. Rev. Pharmacol. Toxicol. 30: 169–195

Okuda, H. (1991) Esterases (including choline esterases). In: Kuby, S. A. (ed.) A Study of Enzymes. Vol. II. Mechanism of Enzyme Action. CRC Press, Florida, pp 563–577

Potter, D. W., Hinson, J. A. (1986) Reactions of *N*-acetyl-*p*-benzoquinone imines with reduced glutathione, acetaminophen, and NADPH. Mol. Pharmacol. 30: 33–41

Potter, D. W., Hinson, J. A. (1987) Mechanisms of acetaminophen oxidation to *N*-acetyl-*p*-benzoquinone imine by horse-radish peroxidases and cytochrome P-450. J. Biol. Chem. 262: 966–973

Satoh, H., Martin, B. M., Schulick, A. H., Christ, D. D., Kenna, J. G., Pohl, L. R. (1989) Human anti-endoplasmic reticulum antibodies in sera of patients with halothane-induced hepatitis are directed against a trifluoroacetylated carboxylesterase. Proc. Natl. Acad. Sci. USA 86: 322–326

Satoh, T. (1987) Role of carboxylesterases in xenobiotic metabolism. Rev. Biochem. Toxicol. 8: 155–181

Talcott, R. E. (1979) Hepatic and extrahepatic malathion carboxylesterases. Assay and localization in the rat. Toxicol. Appl. Pharmacol. 47: 145–150

Talcott, R. E., Ketterman, A., Harger, W., Denk, H., Kerjaschki, D., Zeiler, I., Eckerstorfer, R. (1980) Microsomal lipid peroxidation: catalysis, effects, and inhibition by cytosolic protein. In: Coon, M. J., Cooney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., O'Brien, R. J. (eds) Microsomes, Drug Oxidations, and Chemical Carcinogenesis. Vol. II. Academic Press, New York, pp 753–761

Talcott, R. E., Pond, S. M., Ketterman, A., Becker, C. E. (1982) Ethylesterases as indicators of liver damage. I. Studies on malathion carboxylesterase. Toxicol. Appl. Pharmacol. 65: 69–74