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MEPERIDINE CARBOXYLESTERASE IN MOUSE AND HUMAN LIVERS*

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Abstract—Meperidine carboxylesterase activity was assayed in subcellular fractions of mouse and human liver by coupling the hydrolytic production of ethanol to the reduction of a tetrazolium dye. In mouse liver, the activity was found to be distributed among the mitochondrial, light mitochondrial, and microsomal fractions, whereas in human liver activity was found only in the microsomal fraction. The meperidine carboxylesterases in mouse liver and human liver were inhibited by two irreversible serine hydrolase inactivators (diisopropyl fluorophosphate and paraoxon) and by a reversible transition state analog (trifluoromercaptophenylacetone). Compared to the activities in mouse and human liver microsomes, the activity in mouse liver mitochondria was highly sensitive to the three inhibitors.

Meperidine (Pethidine is the international non-proprietary name) is a synthetic opioid-like drug, widely used as an analgesic agent [1]. The duration and intensity of action of opiates in experimental animals and humans are chiefly limited by the extensive metabolism which these drugs undergo in the body [2]. The main metabolic pathway of meperidine in man is the hydrolysis of the carboxy ethyl ester and the formation of meperidinic acid which is, in turn, partially conjugated [1–3]. Meperidine is also metabolized to normeperidine and about one-third of administered drug can be accounted for, in the urine, as N-demethylated derivatives [1–4]. Normeperidine is pharmacologically active [5] and may cause seizures in humans [6]. Minor metabolites have been also identified including meperidine *N*-oxide [7], parahydroxymeperidine [8], and a hydroxymethoxy derivative of meperidine [9]. A similar metabolic pattern has been reported in mice [10].

Meperidine carboxylesterase (MCE‖) activity has been found in the livers of several mammals including man, dog, rat, guinea pig and rabbit [11], but the hydrolytic enzymes have not been well purified [12] and little is known about their biochemical properties. In this report, we describe an assay procedure that may facilitate the study of MCE and also present initial data from a comparative study of the enzymes in the mouse and human liver fractions.

MATERIALS AND METHODS

Chemicals. Paraoxon (*0,0*-diethyl-4-nitrophenyl phosphate) was purchased from the Aldrich Chemical Co., Inc., Milwaukee, WI. It was cleaned (several inhibitory impurities are often present) by twice washing a solution in trichloroethylene with bicarbonate solution followed by two washes in water, drying over sodium sulfate, and removing the solvent *in vacuo*. Stock solutions were in dry acetone. Diisopropyl fluorophosphate (DFP), alcohol dehydrogenase (ADH), NAD, DT-diaphorase ("NAD-diaphorase"), *p*-iodonitrotetrazolium violet (INT), and Tris buffer (Trizma) were purchased from the Sigma Chemical Co., St. Louis, MO. 1,1,1-Trifluoro-3-mercaptophenylacetone was a gift of Dr. B. Hammock (University of California, Davis). Meperidine hydrochloride was a gift from the Sterling–Winthrop Research Institute, Rensselaer, NY. All other chemicals were analytical reagent grade.

Tissue source and preparation. Mouse livers were obtained from Swiss–Webster mice (4–6 weeks of age) killed by cervical translocation. Livers were perfused with cold KCl, 1.15%. Human liver samples (1–5 g) were obtained from seven patients undergoing abdominal surgery and were normal in histologic appearance. The samples were frozen at −80° immediately upon removal from the patient and stored until assayed. Liver homogenates (20% in 1.15% KCl) were prepared with a Teflon–glass motor-driven homogenizer, and subcellular fractions were isolated by differential centrifugation [13]. For the studies on substrate affinity and inhibition potency, a pool of livers was homogenized, and the separated fractions were stored at −80° until assayed. MCE activity was found to be stable over 2–3 months.

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‖ Abbreviations: MCE, meperidine carbpxylesterase and DFP, diisopropyl fluorophosphate.

MCE assays. MCE activity was measured in all fractions by coupling the release of ethanol to the reduction of a tetrazolium dye as described for the colorimetric assay* of malathion carboxylesterase [14]. A Kontron model 810 UV-VIS spectrophotometer was used to monitor meperidine hydrolysis. Microsomal contaminations of the mitochondrial and light mitochondrial fractions were assayed by determining the concentrations of cytochrome P-450 in these fractions [15]. Protein was measured according to Lowry *et al.* [16]. A final meperidine concentration of 1 mM and a final protein concentration of 0.5 mg/ml were used for measurements of the specific activity of each tissue fraction. For determination of Michaelis constants, concentrations of 0.125, 0.5, 0.75, 1 and 1.5 mM meperidine were used. The Michaelis constants were estimated with Lineweaver–Burk plots. All assays were done at 37° in 2.5 ml of 100 mM Tris–HCl buffer, pH 7.4. Apparent optimal pH and temperature were found to be 7.4 to 8.0 and 37° for the mitochondrial MCE. These optima can be differentiated from those of the assay system, and from those previously observed in rat or rabbit liver microsomes [12, 17].

Inhibition studies. Three serine hydrolase inhibitors, paraoxon, DFP, and 1,1,1-trifluoro-3-mercaptophenylacetone, were selected for study as inhibitors of MCE. Experiments were designed to provide estimates of the concentrations required to inhibit 50% of the enzyme activity present in mouse liver microsomes, mouse liver mitochondria, and human liver microsomes. The tissue fractions were suspended in 100 mM Tris–HCl buffer to a final protein concentration of approximately 0.5 mg/ml; 2.5-ml portions were then pipetted into separate flasks to which various concentrations of inhibitor were added. In the experiments with the progressive, time-dependent enzyme inactivators (paraoxon and DFP), the tissues were preincubated with the inhibitors for 20 min at 37° before the residual MCE was measured. In the experiments with 1,1,1-trifluoro-3-mercaptophenylacetone, a non-progressive inhibitor, MCE activity was measured immediately after addition of the inhibitor. In all experiments, the I_{50} values were calculated by plotting the logarithm of the percentage of total activity remaining versus the concentration of the inhibitor [18].

It was of interest to determine whether or not the effects of 1,1,1-trifluoro-3-mercaptophenylacetone were reversible. In a typical experiment, graded concentrations of the inhibitor were added to a series of tubes containing 5 ml of protein suspension, 0.5 mg/ml of MCE assay mixture. An aliquot (2.5 ml) was withdrawn from each tube and assayed for MCE activity; the remaining 2.5-ml suspensions were centrifuged. The pellets were resuspended in equal volumes of inhibitor-free assay mixture and assayed for MCE.

* In this system, color production is dependent on the presence of alcohol dehydrogenase in the assay mixture, indicating that INT reduction results from ethanol production. Beer's law was found to apply for ethanol concentrations between 0 and 75 μM.

Table 1. MCE activity in the post-3500 *g* fractions of mouse and human liver*

Subcellular fraction	MCE specific activity Mouse	Human
Light mitochondria and lysosomes	1.50 ± 0.26 (4)	ND†
Mitochondria	1.99 ± 0.59 (4)	ND
Microsomes	1.93 ± 0.23 (4)	1.11 ± 0.07 (3)
Cytosol	ND	ND

* The microsomal contamination of the heavier particulate fractions was estimated by measuring P-450 content [15]; the specific activity of these fractions, tabulated above, was calculated by subtracting the activities due to microsomal contamination from the observed specific activities. Values are means ± S.D. expressed in nmoles per min per mg protein. The number of subjects is given in parentheses.
† Not detectable.

RESULTS

The coupled enzyme assay method used previously to measure malathion carboxylesterase activity was suitable for measuring MCE activities of isolated subcellular liver fractions. The specific MCE activity of whole liver homogenate could not be measured reproducibly with this assay system, because of the relatively low levels of activity, the turbidity of the homogenate, and the presence of interfering reducing equivalents. The specific MCE activities of mouse liver and human liver post-3500 *g* fractions are reported in Table 1. In human liver, only the microsomal fraction contained detectable levels of activity, but in mouse liver the enzyme was present in all particulate fractions, at about the same specific activity level, after corrections were made for microsomal contamination of the heavier fractions.

Apparent Michaelis constants were determined for the MCE activities associated with mouse liver

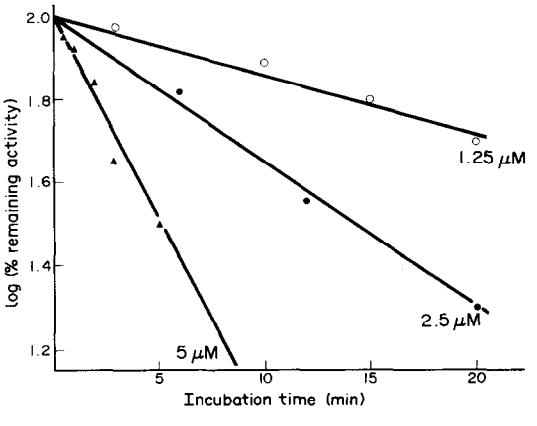

Fig. 1. Time course of inhibition of mouse liver microsomal MCE by paraoxon at different concentrations (1.25, 2.5 and 5.0 μM). The enzyme was inhibited at pH 7.4 and 37°, and the remaining activity was measured under identical conditions in the absence of inhibitor. Inhibition was continued up to the 100% level (not shown in the figure).

Table 2. Effects of substrate and inhibitor concentrations on liver meperidine carboxylesterase activities*

	Meperidine		Interaction of MCE and: DFP	Paraoxon	1,1,1-Trifluoro-3-mercaptophenylacetone
Subcellular fraction	K_m (μM)	V_{max} [nmoles° min^{-1}°(mg protein)$^{-1}$]	I_{50} (μM)	I_{50} (μM)	I_{50}(μM)
Mouse liver mitochondria	230	1.97	0.15	0.30	0.05
Mouse liver microsomes	180	2.32	0.50	1.20	0.50
Human liver microsomes	450	1.40	0.50	4.00	0.20

* Graded concentrations of meperidine were used to determine the Michaelis constants. Graded concentrations of the organophosphates DFP and paraoxon were incubated with tissue for 20 min at 37° before measuring the residual MCE activities. Graded concentrations of 1,1,1-trifluoro-3-mercaptophenylacetone were added to tissue suspensions immediately before measuring MCE activities.

microsomes, mouse liver mitochondria, and human liver microsomes (Table 2). The K_m and V_{max} values were within the same order of magnitude, ranging from 180 to 450 μM and 1.4 to 2.3 nmoles per min per mg protein respectively.

Inhibition of MCE activities by organophosphate was progressive and time dependent. A typical experiment is shown in Fig. 1. The most sensitive activity was associated with the mouse liver mitochondria (Table 2); estimated concentrations of 50 mM 1,1,1-trifluoro-3-mercaptophenylacetone, 150 nM DFP, and 300 nM paraoxon were sufficient to inhibit or inactivate 50% of this MCE activity. Most of the inhibition produced by 1,1,1-trifluoro-3-mercaptophenylacetone could be reversed by a single washing of the particulate fraction. The results of one representative experiment are shown in Fig. 2.

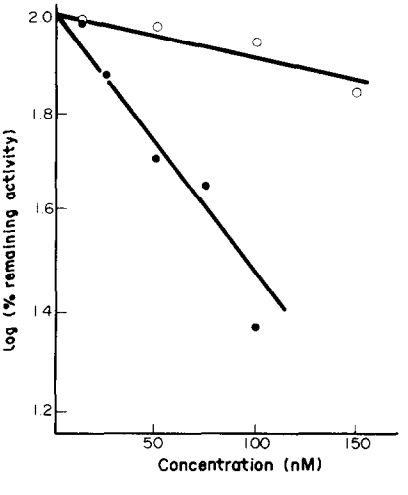

Fig. 2. Effect of washing on the inhibition of MCE activity by 1,1,1-trifluoro-3-mercaptophenylacetone. Graded concentrations of inhibitor were added to buffered suspensions of mouse liver mitochondria, pH 7.4, at 37°. Aliquots of the mitochondria were either assayed for residual MCE activity immediately (●) or were diluted 70-fold, centrifuged, resuspended in the original volume with fresh buffer, and then assayed (○). Negative controls were performed with the enzyme inhibited by paraoxon (0.1 and 0.3 μM).

DISCUSSION

Previously, the meperidine carboxylesterase activity of rat liver microsomes had been measured by determining the amount of ethanol released from meperidine at the end of a 60-minute incubation period [19]. This assay procedure is probably adequate for measuring the hydrolysis rate if the initial meperidine concentration is well in excess of the K_m but, if lesser concentrations are present initially (as in the determination of Michaelis constants), it may not yield accurate estimations of the true initial velocities. Therefore, we have added alcohol dehydrogenase, NAD, DT-diaphorase, and INT to the assay mixture in order to couple the hydrolytic step to the reduction of a tetrazolium dye. With this assay system, meperidine hydrolysis could be recorded continuously from the time that the substrate was added to the sample cuvette. From the recordings, initial velocities could be estimated readily, regardless of whether the initial substrate concentrations were saturating or sub-saturating.

Numerous differential centrifugation experiments have shown that nonspecific hepatic carboxylesterases are localized predominantly in the microsomes [11]. In human liver, the subcellular distribution of MCE activity was typical but, in mouse liver, the distribution showed significant amounts of activity in the mitochondrial fractions. This finding suggests that, in the mouse, the liver mitochondria may be an important site of meperidine hydrolysis. It also suggests that other drugs may be hydrolyzed in the liver mitochondria of mice, because drug-metabolizing carboxylesterases are rather nonspecific and

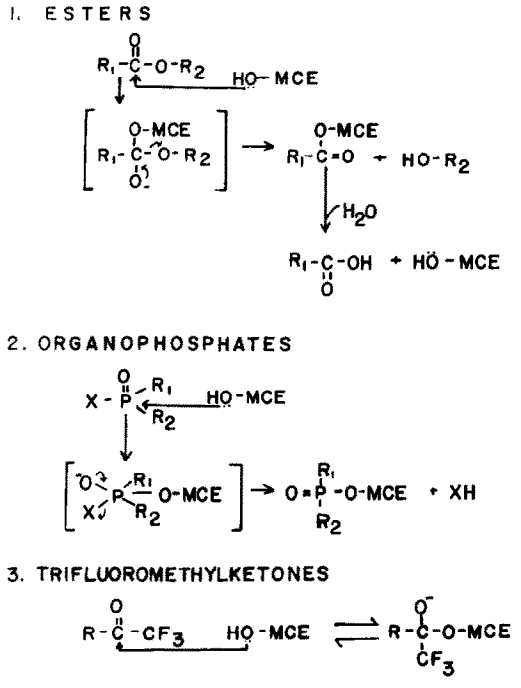

Fig. 3. Association of MCE with compounds that have electrophilic centers. The mechanisms by which serine hydrolases are believed to react with substrates and inhibitors are depicted above. Ester hydrolysis is thought to proceed through a tetrahedral transition state complex that is mimicked by the hemiketal structures formed by trifluoromethylketones [21]. Organophosphates react stoichiometrically with the catalytic sites of serine hydrolases, phosphorylating the enzymes. The MCE activities investigated in this study were inhibited by low concentrations of two organophosphates and one trifluoromethylketone, strongly suggesting that they are serine hydrolases.

exist in isoenzymic forms [11]. Moreover, Markert and Hunter [20] using histochemical techniques revealed that a high percentage of mouse liver nonspecific carboxylesterase activity resides in the mitochondria. While it remains to be determined whether the mouse liver mitochondrial meperidine carboxylesterases function *in vivo*, it is interesting to note that the analgesic dose of meperidine in mice [5], on a body weight basis, appears to be several-fold higher than the analgesic dose in humans [1]. In view of the inverse relationship between analgesic potency and *in vivo* hydrolysis [10], it is certainly possible that extramicrosomal MCE, present in mouse liver but not in human liver, may be one of the factors contributing to the relative resistance of mice to meperidine-induced analgesia.

Typically, drug-metabolizing carboxylesterases are serine hydrolases (EC 3.1.1.1.) and, as such, associate with the electrophilic moieties of esters or amides, organophosphates [11], and trifluoromethylketones [21]. The details of these interactions are depicted in Fig. 3. The MCE activities present in mouse liver mitochondria, mouse liver microsomes, and human liver microsomes were sensitive to organophosphate and trifluoromethylketone inhibition, suggesting that serine hydrolases are the catalytic species.

It remains to be determined whether or not the microsomal and mitochondrial MCE are structurally identical. The strongest evidence for structural non-identity is the order-of-magnitude difference in sensitivity to 1,1,1-trifluoro-3-mercaptophenylacetone. However, it is possible that the membrane matrix may affect inhibitor–enzyme association and the degree of nonspecific binding. Solubilization and purification of MCE will be necessary for definitive characterization studies but, to date, MCEs have not been purified successfully [11]. In this regard, the findings of the 1,1,1,-trifluoro-3-mercaptophenylacetone inhibition studies may be significant. Analogs of this potent, reversible inhibitor attached to insoluble supports may be useful ligands for the affinity chromatography of solubilized MCE.

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