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Research Article

Hydrolysis of Carbonates, Thiocarbonates, Carbamates, and Carboxylic Esters of α-Naphthol, β-Naphthol, and *p*-Nitrophenol by Human, Rat, and Mouse Liver Carboxylesterases

Tien L. Huang,[1,2] András Székács,[1,3] Tamon Uematsu,[1,4] Eiichi Kuwano,[1,5] Andrew Parkinson,[6] and Bruce D. Hammock[1,7]

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Thirty carbonates, thiocarbonates, carbamates, and carboxylic esters of α-naphthol, β-naphthol, and *p*-nitrophenol were synthesized and tested as substrates for liver carboxylesterases from the crude microsomal fractions of human and mouse, and purified isozymes, hydrolases A and B, from rat liver microsomes. The carbonates, thiocarbonates, and carboxylic esters of α-naphthol were cleaved more rapidly than the corresponding β-naphthol isomers by the mammalian liver esterases. α-Naphthyl esters of acetic, propionic, and butyric acids were among the best substrates tested for these enzymes. The majority of the substrates was consistently hydrolyzed at higher rates by hydrolase B compared with hydrolase A, although the Michaelis–Menten constant (K_m) values of selected substrates differed widely with these two isozymes. Malathion was a 15-fold better substrate for hydrolase B than for hydrolase A. Compared with the corresponding carboxylates, the carbonate moiety of α- and β-naphthol and *p*-nitrophenol lowered the specific activities of the enzymes by about fivefold but improved stability under basic conditions. The optimum pH of mouse liver esterase with the acetate, methylcarbonate, and ethylthiocarbonate of α-naphthol was between pH 7.0 and pH 7.6. Human and mouse liver microsomal esterase activities were about five orders of magnitude lower than the esterase activities of purified rat liver hydrolase B. A relationship between the catalytic activity of the enzymes and the lipophilicity of the naphthyl substrates indicated that (i) in the α- and β-naphthyl carbonate series, an inverse relationship between enzyme activity and lipophilicity of the substrates was observed, whereas (ii) in the α-naphthyl carboxylate series, an increase in enzyme activity with increasing lipophilicity of the substrates up to a log*P* value of about 4.0 was observed, after which the enzyme activity decreased.

KEY WORDS: carboxylesterases; mammalian liver; hydrolases A and B; α- and β-naphthyl substrates; *p*-nitrophenol substrates; esterase assay, in microtiter plate.

INTRODUCTION

The carboxylesterases (EC 3.1.1.1) constitute a heterogeneous group of isozymes that can catalyze the hydrolysis of a wide range of esters, amides and thioesters. Therefore, they play an important role in the metabolism of drugs and lipids (1,2). However, in spite of a plethora of literature on the analysis of esterases, their physiological functions in many cases are not known.

Carboxylic esters, such as the esters of naphthols and nitrophenols, are commonly used to measure esterase activity. In contrast to the commonly used carboxylic esters, which have only one functional group, organic carbonates and carbonate derivatives are bifunctional. This offers the advantage of having two possible leaving groups in the molecule. The order in which the two leaving groups of asymmetrical carbonates leave the molecule during esterolytic hydrolysis can provide a better understanding of the mode of substrate binding to the active site of the enzyme. In addition, important information on the substrate specificity of carboxylesterases can be obtained by using a variety of substrates of different chemical classes.

Organic carbonates have been tested as analogous substrates for esterases. Choline carbonates were found to be cleaved by acetylcholinesterase (EC 3.1.1.7) (3). Fife *et al.*

[1] Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616.
[2] College of Pharmacy, Xavier University of Louisiana, New Orleans, Louisiana 70125.
[3] Plant Protection Institute of the Hungarian Academy of Sciences, P.O. Box 102, 1525 Budapest, Hungary.
[4] Sumitomo Chemical Co., Takarazuka 665, Japan.
[5] Department of Agricultural Chemistry, Kyushu University, Fukuoka 812, Japan.
[6] Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas 66160-7417.
[7] To whom correspondence should be addressed at Department of Entomology, University of California, Davis, California 95616.

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(4) have investigated the chemical and α-chymotypsin-catalyzed hydrolysis of cyclic and acyclic nitrophenyl carbonates [e.g., bis(4-nitrophenyl)carbonate and *O*-(4-nitrophenylene)carbonate]. The metabolic hydrolysis of the acaricide dinobuton (2-sec-butyl-4,6-dinitrophenyl isopropyl carbonate) by esterases produces an active toxin. Its production is directly correlated with serum esterase levels in mammals (5). The esterolytic cleavage of several carbonate drug precursors, e.g., carbonates of acetaminophen (6) and salicyclic acid (7), by esterases from various species has been successfully applied in prodrug approaches. To study substrate specificity adequately it will be important to purify, clone, and express hepatic esterases involved in the degradation of compounds of pharmacological and toxicological interest. However, the purification efforts to date have used a very limited number of surrogate substrates for monitoring esterase activity. Before initiating work on the affinity purification of hepatic esterases, it seemed appropriate to characterize a library of surrogate substrates.

Naphthyl esters, namely, naphthyl acetates, are often used as diagnostic substrates for esterolytic enzymes. The hydrolysis product of these substrates, naphthol, can form nondiffusible color stains with diazonium salts (fast blue) that can be sensitively detected on polyacrylamide gel electrophoresis and also provide a simple continuous colorimetric assay for measuring esterase activity. The substrates reported in this study are useful in characterizing the substrate specificities of the various isozymes of carboxylesterases. Thus, we synthesized a series of carbonates, thiocarbonates, carbamates, and carboxylic esters containing α- or β-naphthol or *p*-nitrophenol as leaving groups and tested them as substrates of human, rat, and mouse liver microsomal esterases. Although the compounds have very different physical properties from the parent naphthyl esters, naphthol (or *p*-nitrophenol) is the product of their hydrolysis simplifying analysis.

MATERIALS AND METHODS

Chemicals

α-Naphthol, α-naphthyl acetate, α-naphthyl propionate, α-naphthyl butyrate, and β-naphthyl acetate were purchased from Sigma Chemical Co. (St. Louis, MO). Protein dye reagent was obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals were purchased from Aldrich Chemical Co., (Milwaukee, WI). The commercial chemicals and solvents used were of the best grade available.

Analytical Procedures

All reported melting points are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were recorded on a Varian EM-390 (90 MHz) spectrometer with carbon tetrachloride or deuterated chloroform ($CDCl_3$) as a solvent and tetramethylsilane as the internal standard. Elemental analyses were performed at the Berkeley Analytical Laboratory of the University of California, Berkeley, CA. The purity of the compounds was determined by thin-layer chromatography (TLC) in multiple solvent systems.

Synthesis of Substrates

The chemical structures of the synthesized substrates used in this study are shown in Tables I and II. The *O*-alkyl carbonates of α-naphthol, β-naphthol, and *p*-nitrophenol were prepared according to published procedures (8,9) by acylation of the naphthols or *p*-nitrophenol with the appropriate chloroformates. The *S*-ethyl thiocarbonates of α- and β-naphthols were synthesized by reacting the naphthols with *S*-ethyl chlorothioformate (10). *S*-Methyl thiocarbonate of *p*-nitrophenol was synthesized by reacting *p*-nitrophenyl-chloroformate with sodium thiomethoxide in dioxane. Carbamates of α-naphthol and *p*-nitrophenol were prepared by the conventional method (11) of reacting the anhydrous α-naphthol or *p*-nitrophenol with the appropriate alkyl isocyanate at room temperature. The carboxylic esters of α- and β-naphthol and *p*-nitrophenol were either available commercially or prepared by reacting the naphthols or *p*-nitrophenol with the dicyclohexylcarbodiimide-activated alkyl carboxylic acids. The compounds yielded a single spot on thin-layer chromatography (TLC), and their purity was tested in at least two solvent systems. Representative synthetic procedures are described for compounds **13**, **25**, **34**, and **35**.

α-Naphthyl 2,3-Epoxy-3-Phenylpropyl Carbonate (13)

Pyridine (3.2 g, 40 mmol) was added dropwise to a cooled (ice bath) solution of cinnamyl alcohol (3.2 g, 24 mmol) and α-naphthyl chloroformate (5 g, 24 mmol) in ether (30 mL). After stirring at room temperature for 5 hr, the ether solution was washed with brine and dried over Na_2SO_4. After removal of the solvent, the residue was chromatographed on silica gel with hexane–ethyl acetate (3:1, v/v). Concentration under reduced pressure yielded 6.5 g (89%) of α-naphthyl cinnamyl carbonate. ^{1}H NMR ($CDCl_3$) δ: 4.80 (2H, d, J = 6 Hz), 6.2–6.9 (2H, m), 7.1–8.2 (12H, m).

To a cooled (ice bath) solution of α-naphthyl cinnamyl carbonate (4.0 g, 13 mmol) in dichloromethane (30 mL) was added 85% of *m*-chloroperbenzoic acid (3.4 g, 17 mmol). After stirring for 18 hr at room temperature, the dichloromethane solution was washed with 5% $NaHCO_3$, brine, and dried over Na_2SO_4. The solvent was removed and the residue was recrystallized from ethanol to afford 3.0 g (71%) of **13**, mp 104–105°C. ^{1}H NMR ($CDCl_3$) δ: 3.3–3.5 (1H, m), 3.9 (1H, d, J = 2 Hz), 4.40 (1H, dd, J_a = 5 Hz, J_b = 12 Hz), 4.72 (1H, dd, J_a = 3 Hz, J_b = 12 Hz), 7.1–8.1 (12H, m). *Anal.* Found: C, 74.78; H, 5.01. Calcd. for $C_{20}H_{16}O_4$: C, 75.00; H, 5.00.

α-Naphthyl 4-Phenyl-3,4-Epoxybutanoate (25)

Dicyclohexylcarbodiimide (2.1 g, 10 mmol) was added to a cooled (ice bath) solution of 4-phenyl-3-butenoic acid (1.6 g, 9.9 mmol), α-naphthol (1.4 g, 9.7 mmol), and dimethylaminopyridine (0.10 g, 0.82 mmol) in dichloromethane (50 mL). The reaction mixture was stirred overnight at room temperature and the precipitate was removed by filtration. The filtrate was washed with 5% HCl, 5% Na_2CO_3, and brine and dried over Na_2SO_4. The solvent was removed and the residue chromatographed on silica gel with hexane–ethyl acetate (6:1, v/v). The eluate was concentrated under reduced

pressure and recrystallized from ethanol, yielding 1.75 g (62%) of α-naphthyl 4-phenyl-3-butenoate, mp 72–73°C. ^{1}H NMR δ: 3.63 (2H, d, J = 6 Hz), 6.3–6.8 (2H, m), 7.2–8.0 (12H, m).

α-Naphthyl 4-phenyl-3-butenoate was epoxidized according to the procedure described for compound **13**. The yield of **25** was 0.91 g (62%), mp 64–65°C. NMR δ: 3.15 (2H, d, J = 6 Hz), 3.5–3.7 (1H, m), 3.92 (1H, d, J = 2 Hz), 7.3–8.1 (12H, m). *Anal.* Found: C, 78.94; H, 5.29. Calcd. for $C_{20}H_{16}O_3$: C, 78.95; H, 5.26.

p-*Nitrophenyl Methylthioformate (34)*

p-Nitrophenyl chloroformate (1.0 g, 5.0 mmol) was added to a stirred suspension of sodium thiomethoxide (0.35 g, 5.0 mmol) in 1,4-dioxane (30 mL) at room temperature under a nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. 1,4-Dioxane was evaporated, water (25 mL) was added to the residue, and the product was extracted with ethyl acetate (20 mL × 3). The organic layer was washed with brine, dried over $MgSO_4$, and then evaporated to give an oily residue which was purified by silica gel column chromatography with hexane–ethyl acetate (5:1, v/v). Concentration under reduced pressure yielded 0.94 g (88%) of **34**. ^{1}H NMR ($CDCl_3$) δ: 2.45 (3H, s), 7.35 (2H, d), 8.25 (2H, d).

p-*Nitrophenyl Methyl Carbamate (35)*

Methyl isocyanate (0.57 g, 10 mmol) was added dropwise to a solution of *p*-nitrophenol (1.4 g, 10 mmol) in anhydrous THF (50 mL) containing catalytic amount of triethylamine. The mixture was stirred for 5 hr and the organic solvent was then evaporated *in vacuo*. The crude product was purified by silica gel column chromatography with hexane–ethyl acetate (5:1, v/v). Concentration under reduced pressure yielded 1.95 g (99%) of **35**. ^{1}H NMR ($CDCl_3$) δ: 2.95 (3H, d), 5.15 (1H, broad), 7.35 (2H, d), 8.3 (2H, d).

Calculation of log*P*

Octanol/water partition coefficients (log*P*) were calculated by the FRAGMENT method of Hansch and Leo (12). Reference values for log*P* were obtained from the literature for α-naphthol (log*P* = 2.71) (13,14), for β-naphthol (log*P* = 2.81) (13,14), for benzyl alcohol (log*P* = 1.10) (15), for fluorene (log*P* = 4.18) (12), and for *O*-(1-naphthyl), *N*-methyl carbamate (log*P* = 2.35) (16). Other substituent fragment constants were complied from standard tables (12,17). The fragment constants for carbonate (log*P* = −1.63) and thiolcarbonate (log*P* = −0.62) groups were calculated from their 8-quinoline derivatives (12), from the reference value (log*P* = 1.97) for 8-quinolinol (18). If solvent/water partition coefficient values were available for solutes other than octanol, log*P* for octanol/water was estimated by solvent regression equations (19). It should be remembered that the fragment method can lead to overestimations of log*P* for highly lipophilic compounds.

Enzyme Preparation

The procedure used for the isolation of human and murine liver microsomes was previously described (20). Male Swiss–Webster mice (25–30 g) were purchased from Bantin–Kingman (Fremont, CA). Human liver samples were obtained from the Stanford Research Institute organ bank, Menlo Park, CA, and were stored at −80°C until needed. The livers were from either male or female donors who died of traumatic injury or stroke. All livers used tested negative for hepatitis and HIV-1. In brief, the liver homogenates (12.5% of liver weight, w/v) were centrifuged at 10,000*g* for 10 min, and the supernatant fractions were decanted and centrifuged at 100,000*g* for 1 hr to obtain the microsomal pellet. The pellet was washed once with 0.25 *M* sucrose–0.05 *M* sodium phosphate buffer (pH 7.4), resuspended in the same buffer, and stored at −70°C until used for enzyme assay. Purified rat liver isozymes, hydrolases A and B, were obtained from previous studies (21).

Enzyme Assays

The esterase activity was measured in a continuous assay with 96-well microtiter plates (Dynatech Laboratories, Inc., Virginia, VA) with a Vmax plate reader (Molecular Devices, Palo Alto, CA) as described earlier (22). The associated Softmax software provided with the instrument was used to calculate the standard curves of *p*-nitrophenol and α- and β-naphthol conjugated to the diazonium salt Fast Blue RR, rate of product formation, and protein concentrations used in the assay.

The standard curves of α- and β-naphthol concentrations conjugated to the diazonium salt were determined by adding a series of freshly prepared concentrations of α- and β-naphthol (in THF) to a 0.025% Fast Blue RR salt solution prepared in 0.1 *M* sodium phosphate, pH 7.4. At each concentration, 300 μL of the same preparation was added to four wells and the absorbance at 450 nm was recorded. The final THF concentration was less than 1%.

For measuring the rates of hydrolysis of the naphthyl substrates by the mammalian liver esterases, 20 μL of the enzyme solution in 0.1 *M* sodium phosphate buffer, pH 7.4, was added to wells containing 278 μL of a 0.025% Fast Blue RR salt solution prepared in the same buffer. In the assay of hydrolases A and B, the buffer also contained 50 μg/mL bovine serum albumin to stabilize the purified enzymes. In the nonenzymatic reaction, 20 μL buffer instead of the enzyme solution was added. The reaction was started by the injection of 2 μL of the substrates (in THF) to give a final concentration of 5×10^{-4} *M*. The samples were immediately mixed with the automix mode on the plate reader and the hydrolytic rates were monitored at 450 nm for 1–5 min at 23°C. It should be pointed out that the product of substrate hydrolysis (i.e., naphthol) reacts instantaneously with the reagent Fast Blue RR under these conditions (22). Therefore, the hydrolysis of the naphthyl substrates is the rate-limiting step in the reaction leading to chromophore formation. Separate experiments indicated that up to 5 μL of acetone or THF had little effect on enzyme activity.

The hydrolysis of the *p*-nitrophenyl derivatives was determined as described previously (8,20). The incubation mixture contained enzyme solution in 298 μL of 0.1 *M* sodium phosphate buffer, pH 7.4. The reaction was started by the injection of 2 μL of the substrates (in acetone) to give a final concentration of 5×10^{-4} *M*. The liberation of *p*-nitrophenol was monitored for 2 min at 405 nm.

Table I. Specific Activities and log*P* Values for α- and β-Naphthyl Derivatives as Spectrophotometric Substrates of Crude Human and Mouse Liver Microsomes and Purified Hydrolases A and B from Rat Liver Microsomes[a]

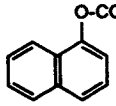
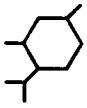
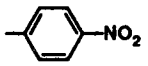
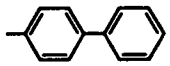
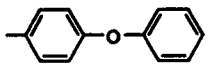
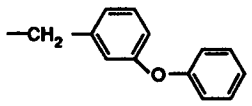
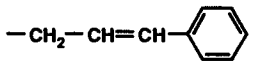
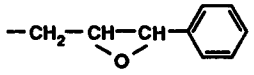
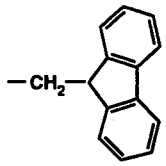
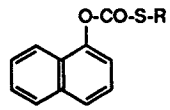
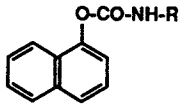
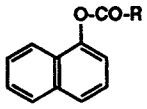
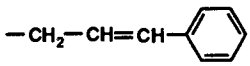
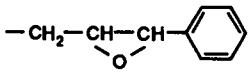
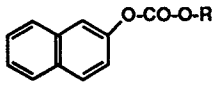
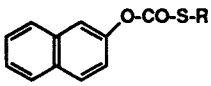
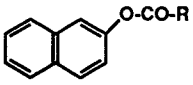
Substrate no.	Structures of substrates	Sp act (μmol/min/mg protein)				log*P*
		Human	Mouse	Hydrolase A	Hydrolase B	
I. α-Naphthyl carbonates						
1	*R* = $-CH_3$	1.07 ± 0.02	1.04 ± 0.04	15.3 ± 0.07	85.3 ± 4.50	2.32
2	$-CH_2CH_3$	0.90 ± 0.02	0.82 ± 0.04	22.1 ± 0.82	68.3 ± 2.46	2.86
3	$-CH=CH_2$	0.85 ± 0.03	1.63 ± 0.02	84.2 ± 6.45	75.3 ± 4.11	1.67
4	$-CH_2-CH=CH_2$	0.64 ± 0.02	0.66 ± 0.01	18.4 ± 0.47	50.0 ± 3.07	2.62
5	$-CH_2-CH(CH_3)_2$	0.40 ± 0.01	0.23 ± 0.02	2.14 ± 0.06	18.7 ± 0.21	3.68
6		0.33 ± 0.02	ND[b]	NH[c]	NH	4.32
7		0.20 ± 0.02	ND	ND	ND	3.33
8		0.17 ± 0.01	0.16 ± 0.01	2.77 ± 0.38	5.14 ± 0.01	2.95
9		0.232 ± 0.007	ND	ND	ND	4.88
10		0.153 ± 0.008	0.074 ± 0.009	1.58 ± 0.007	1.03 ± 0.05	5.29
11		0.143 ± 0.002	0.056 ± 0.006	1.00 ± 0.07	1.67 ± 0.04	5.02
12		0.165 ± 0.002	0.326 ± 0.02	ND	ND	4.07
13		0.32 ± 0.02	0.227 ± 0.005	3.42 ± 0.34	11.0 ± 0.90	2.49
14		0.070 ± 0.005	0.018 ± 0.005	2.04 ± 0.14	1.23 ± 0.01	5.11
15	−Cholesteryl	0.003 ± 0.002	NH	NH	NH	10.52
II. α-Naphthyl thiocarbonates						

Table I. Continued

Substrate no.	Structures of substrates	Sp act (μmol/min/mg protein) Human	Mouse	Hydrolase A	Hydrolase B	log*P*
16	*R* = $-CH_3$	0.49 ± 0.01	ND	ND	ND	3.33
17	$-CH_2CH_3$	0.25 ± 0.01	0.212 ± 0.002	5.49 ± 0.54	3.25 ± 0.09	3.87
III. α-Naphthyl carbamates						
18	*R* = $-CH_3$	0.002 ± 0.003	0.012 ± 0.001	NH	NH	2.36
19	$-CH_2CH_3$	0.005 ± 0.003	NH	NH	NH	2.90
IV. α-Naphthyl carboxylates						
20	*R* = $-CH_3$	3.89 ± 0.20	3.56 ± 0.03	42.0 ± 1.13	132 ± 10.6	2.77
21	$-CH_2CH_3$	9.28 ± 0.21	3.99 ± 0.13	130 ± 8.6	257 ± 24.9	3.31
22	$-(CH_2)_2CH_3$	14.8 ± 0.17	3.04 ± 0.03	124 ± 6.6	174 ± 11.9	3.85
23	-linoleate	0.006 ± 0.001	NH	NH	0.74 ± 0.05	10.10
24		1.34 ± 0.02	0.38 ± 0.03	8.82 ± 0.46	11.3 ± 0.51	4.52
25		1.84 ± 0.04	0.64 ± 0.01	8.27 ± 0.27	27.1 ± 1.37	2.94
V. β-Naphthyl carbonates						
26	*R* = $-CH_3$	0.094 ± 0.007	0.071 ± 0.005	0.926 ± 0.067	8.62 ± 1.00	2.53
27	$-CH_2CH_3$	0.063 ± 0.005	0.070 ± 0.011	1.06 ± 0.17	7.54 ± 0.58	3.07
28	$-CH_2CH(CH_3)_2$	0.046 ± 0.002	0.045 ± 0.008	NH	2.05 ± 0.07	3.89
VI. β-Naphthyl thiocarbonates						
29	*R* = $-CH_3$	0.059 ± 0.004	ND	ND	ND	3.54
30	$-CH_2CH_3$	0.020 ± 0.002	0.026 ± 0.004	NH	NH	4.08
VII. β-Naphthyl carboxylate						
31	*R* = $-CH_3$	0.311 ± 0.007	0.200 ± 0.005	4.11 ± 0.09	19.5 ± 2.19	2.98
VIII. Other carboxylesterase substrates						
Malathion		ND	0.016 ± 0.001	0.061 ± 0.002	0.920 ± 0.133	
Diethyl succinate		ND	0.178 ± 0.003	2.27 ± 0.07	2.06 ± 0.19	

[a] Assay conditions are described under Materials and Methods. Each value represents the means ± SD of four determinations. Protein concentrations for human liver microsomes, mouse liver microsomes, and hydrolases A and B from rat liver microsomes were 519, 674, 9.84, and 9.87 μg/mL, respectively. The final substrate concentrations were 0.5 m*M*.

[b] Not determined.

[c] No hydrolysis or less than 2×10^{-3} μmol substrate hydrolyzed/min/mg protein.

Table II. Specific Activities for *p*-Nitrophenyl Derivatives as Spectrophotometric Substrates of Crude Mammalian Liver Microsomal Carboxylesterases[a]

Substrate no.	Structures of substrates	Sp act (μmol/min/mg protein) Human	Mouse
32	NO_2-C$_6$H$_4$-O-CO-CH_3	1.32 ± 0.05	3.19 ± 0.15
33	NO_2-C$_6$H$_4$-O-CO-OCH_3	0.25 ± 0.01	0.55 ± 0.02
34	NO_2-C$_6$H$_4$-O-CO-S-CH_3	0.123 ± 0.008	0.103 ± 0.005
35	NO_2-C$_6$H$_4$-O-CO-NH-CH_3	Unstable[b]	Unstable[b]

[a] Assay conditions are described under Materials and Methods. Each value represents the mean ± SD of four determinations. Protein concentrations for human and mouse liver microsomes were 519 and 674 μg/mL, respectively. The final substrate concentrations were 0.5 m*M*.

[b] Rapid spontaneous hydrolysis in 0.1 *M* sodium phosphate buffer, pH 7.4.

The enzyme assays were carried out under conditions where the initial hydrolytic rates were linear with time for the protein and substrate concentrations used. The assay was carried out in quadruplicate. The measured rates were corrected for the spontaneous hydrolysis of the substrate where this was significant. The final concentration of organic solvents (i.e., THF or acetone) used in the assay mixture was less than 1% and caused little or no effect on enzyme activity. The standard curves were used to calculate the esterase activity, expressed as micromoles of α- or β-naphthol or *p*-nitrophenol formed per minute per milligram of protein. Protein concentrations were determined by the Bradford method (23) with bovine serum albumin (fraction V, Sigma) as the standard.

Determination of Kinetic Constants

The Michaelis–Menten parameters, K_m and V_{max}, were calculated based on the computational method of Wilkinson (24). Six to ten substrate solutions of increasing concentrations (2.5–400 μ*M*) in triplicate were incubated with the crude mouse or purified rat isozymes according to the assay conditions described earlier.

Optimum pH of Mouse Liver Esterase

Buffers (278 μL) at pH 6.0–9.0 in 0.2-pH unit increments, were added to 10 μL of murine liver microsomes and incubated at room temperature for 10 min in individual wells. Fast Blue RR salt in 10 μL of distilled water was added to give a final concentration of 0.025% (w/v). The esterase activity was measured after injecting 2 μL of the substrates (in THF) to give a final concentration of 5×10^{-4} *M*. The nonenzymatic spontaneous hydrolysis of substrates **1**, **17**, and **20** selected for this study was also examined in the pH range 6.0–9.0. These nonenzymatic rates were found to be minimal and were subtracted from the reported enzymatic rates. Significant spontaneous (nonenzymatic) hydrolysis of some of these substrates, namely, **20**, was observed only at high pH's (i.e., pH 9.0 and above). The ionic strength of all buffers was 0.1 *M*. A phosphate buffer was used for pH 6.0–8.0 and Tris–HCl for pH 8.0–9.0.

Nonenzymatic Hydrolysis of Selected Substrates

To 150 μL of 0.1 *M* Tris–HCl buffer, pH 9.0 equilibrated at 23°C in a 96-well microtiter plate was added 1 μL of the substrate (5×10^{-4} *M*) in THF. At regular time intervals, 150 μL of 0.1 *M* HCl was added to stop the hydrolysis. The contents in the wells were mixed and 150 μL was pipetted into adjacent wells containing 150 μL of 0.2 *M* sodium phosphate buffer containing 0.050% Fast Blue RR salt. Optical density measurements at 450 nm were corrected for the absorption due to the Fast Blue RR reagent.

Inhibition Studies

Mouse liver microsomal preparations (168 μg/mL) in 0.1 *M* sodium phosphate buffer, pH 7.4, were incubated at room temperature with various concentrations of methyl or ethyl carbamate of α-naphthol. After a 10-min incubation, the rate of α-naphthyl acetate hydrolysis was measured spectrophotometrically as described above.

RESULTS AND DISCUSSION

A number of model substrates such as α-naphthyl acetate and *p*-nitrophenyl acetate are commonly used for studying carboxylesterase activity acting on substrates of toxicological or pharmacological significance. In comparing the structures of malathion (an ester with a small alcohol and a large acid) and α-naphthyl acetate (an ester with a small acid and a large alcohol) (Fig. 1), different classes of carboxylesterases were shown to be involved in the metabolism of both compounds (8,20). Ashour and co-workers (8) reported that in *Drosophila melanogaster*, the major enzyme hydrolyzing malathion was not active on a variety of acetates of α- and β-naphthols. This observation was further supported by inhibition data obtained with substituted trifluoromethylke-

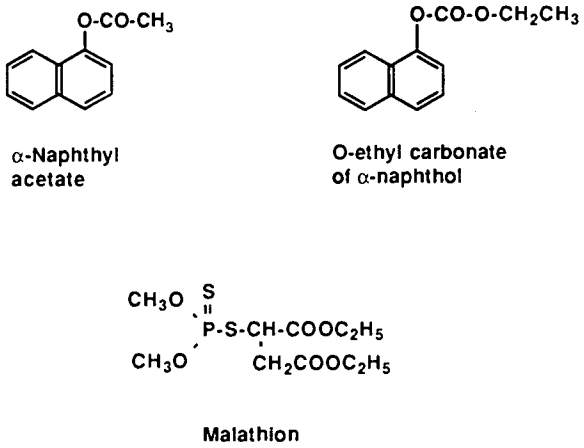


Fig. 1. Comparison of the structures of carboxylesterase substrates.

tones as potent, selective inhibitors of mammalian carboxylesterases which indicated that acetates such as *p*-nitrophenyl acetate and diethyl succinate were hydrolyzed by a different family of carboxylesterases than malathion (20). In the early work on insect juvenile hormone esterase, α-naphthyl acetate was used incorrectly as a substrate to monitor the hydrolysis of juvenile hormone (25). Based on these observations, we synthesized a variety of carbonates which may serve as better model substrates than α-naphthyl acetate since some of these compounds, e.g., *O*-ethyl carbonate of α-naphthol in Fig. 1, can be considered to be an ethyl ester of a large acid like malathion. These carbonates form an unstable carbonic acid following hydrolysis and spontaneously decompose to release carbon dioxide and the chromophore α- or β-naphthol or *p*-nitrophenol. Thus, the same analytical technology developed for α- or β-naphthyl or *p*-nitrophenyl acetates can be applied to the corresponding carbonates.

Substrate Specificities of Liver Microsomal Esterases

The initial rates of hydrolysis of the 35 α- and β-naphthyl and ρ-nitrophenyl derivatives by human, rat, and mouse liver carboxylesterases are shown in Tables I and II. These substrates were also tested on the esterolytic activities of the cytosolic fractions of crude human and mouse liver (data not shown). Liver carboxylesterases are found predominantly in the microsomal fraction and occasionally in the cytosol. The cytosolic esterase activity is usually thought to be the result of autolysis that readily solubilizes the membrane-bound carboxylesterases (1).

The carboxylates of α- and β-naphthol with short acyl groups exhibited the highest rate of hydrolysis by human, rat and mouse liver esterases. α-Naphthyl acetate showed a higher specific activity than β-naphthyl acetate when assayed as substrates for these enzymes. Gomori (26) reported a similar observation wherein human liver and serum esterases were found to hydrolyze esters of α-naphthol much more efficiently than the corresponding β-isomers. In the α-naphthyl carboxylate series, butyrate and propionate were hydrolyzed at the highest rate by human and mouse enzymes, respectively. For rat hydrolases A and B, the highest V_{max} and K_m values were displayed by the butyrate and propionate esters, respectively.

Like the corresponding carboxylates, the carbonates of α-naphthol were hydrolyzed more rapidly than the corresponding carbonates of β-naphthol. Compared to the carboxylates, the carbonate moiety in the α- and β-naphtholic series (Table I) and of *p*-nitrophenol (Table II) lowered the specific activities of the enzymes about fivefold. In the α-naphthyl carbonate series, methyl carbonate was most susceptible to hydrolysis by human and rat hydrolase B, whereas vinyl carbonate was hydrolyzed at the highest rate by rat hydrolase A and mouse liver esterase, respectively. Lengthening or increasing the size of the side chain of the carbonates hindered the enzymatic hydrolysis of these compounds. Thus, the liver microsomal esterases from human, rat, and mouse seemed to be relatively specific for short-chain carboxylic esters and carbonates of α- and β-naphthols.

Replacement of the *O*-ethyl group in the carbonate substrates with the corresponding *S*-ethyl or NH-ethyl groups led to a marked decrease in the rate of hydrolysis of these substrates by the mammalian esterases. The ethyl carbamate of α-naphthol was not hydrolyzed by the mouse and rat esterases. Since the methyl and ethyl carbamates of α-naphthol were extremely poor substrates for the mammalian enzymes, they were tested as inhibitors for the enzymatic hydrolysis of α-naphthyl acetate. As expected, the methyl and ethyl carbamates inhibited mouse liver esterase with an I_{50} of 2.74×10^{-5} and 2.96×10^{-6} *M*, respectively. The strong potency of the carbamates indicates that the compounds bind well to the enzyme by carbamylating the active-site serine residue but are poorly hydrolyzed because of the stability of the carbamylated enzyme–inhibitor complex.

Quantitative Structure–Activity Relationships

The structure–activity relationships between the specific activities of human liver microsomes and rat liver hydrolase B and the lipophilicities of the α- and β-naphthyl series of substrates are illustrated in Fig. 2. The trends appear to be similar with crude esterases solubilized from mouse liver microsomes and rat liver hydrolase A. A generally decreasing trend of enzyme specific activity with lipophilicity has been recorded for α- and β-naphthyl alkyl carbonates and thiocarbonates (Fig. 2). The decrease in the hydrolytic reaction rate with increasing alkyl chain length and hydrophobicity suggests that a lipophilic acyl-enzyme is probably very slow to reactivate as has been shown for phenyl butyrate and other substrates (27,28). It is likely that the naphthyl moiety, being a better leaving group, is cleaved first during the formation of the acyl-enzyme intermediate. The hydrophobic alkyl carbonate moiety, when attached to the region at or near the active site, may not be efficiently removed because of a possible high affinity for the enzyme. This would then result in a decreased turnover rate. On the basis of these considerations, a fall in V_{max} should correlate with a decrease in K_m for the alkyl carbonate series. This is shown for a limited set of compounds in Table III. Comparing the esterase activity, in terms of kinetic parameters, of compound 1 versus 5, the increased binding (lower K_m) of 5 resulted in a lower turnover rate (lower V_{max}) by the esterase enzymes from mouse and rat hydrolases A and B. Optimal hydrolytic activity in the series (Fig. 2) appears with the compounds of low lipophilicities (e.g., compound 1), but even this compound is rather lipophilic due to the nature of the naphthyl moiety. The fact that aliphatic thiocarbonates (compounds 16 and 17) also show the same trend when the data in Fig. 2A are examined by linear regression indicates that the presence of the sulfur does not significantly alter enzymatic hydrolysis and may go through a similar mechanism of hydrolysis.

It was rather surprising that the α-naphthyl aryl carbonates did not fit in the trend of similar aliphatic derivatives. This indicates that elements other than lipophilicity, i.e., steric or electronic factors, are also important for esterolytic action. Unfortunately, the role of such parameters cannot be quantitatively analyzed on the present sample, due to the low number of aromatic analogues. No correlation was observed among the aromatic analogues themselves and they clearly did not fit linear regression lines calculated from the data in Fig. 2. Since none of the aromatic compounds fit in

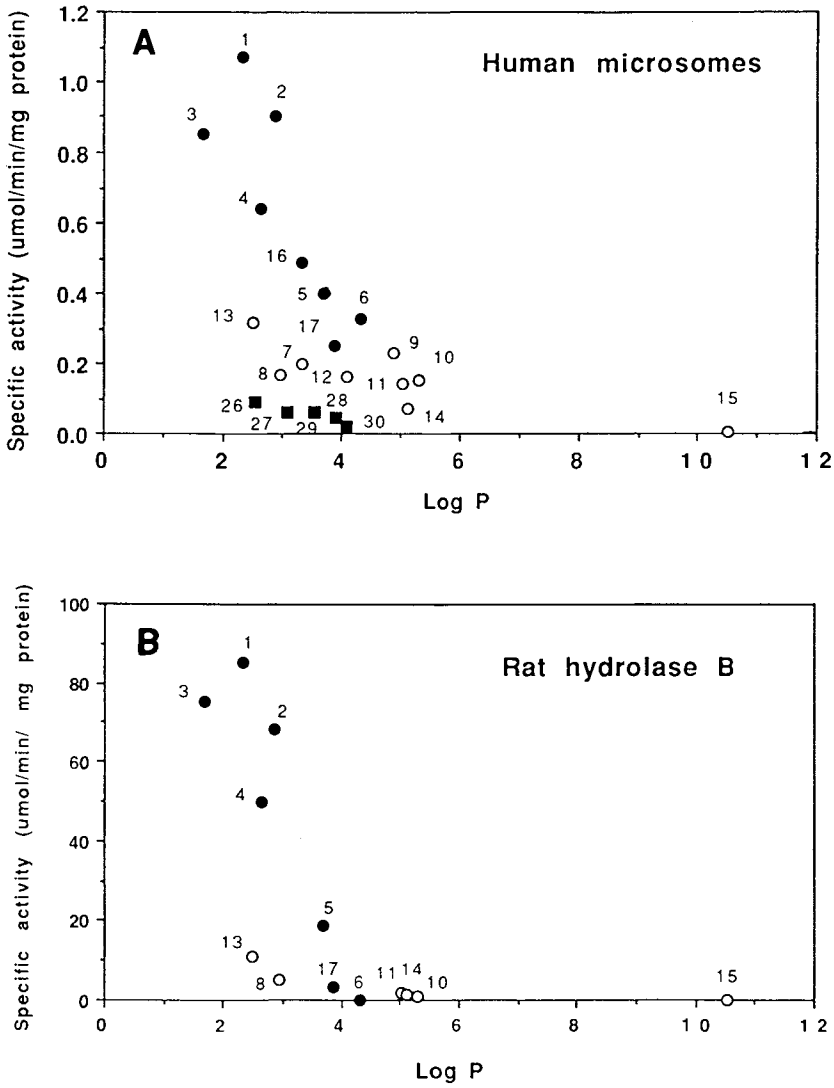


Fig. 2. Dependence of the specific activities of rat hydrolase B and human liver microsomal carboxylesterase on the lipophilicities (log*P*) of aliphatic and aromatic naphthyl carbonates and derivatives. Numbers represent data for compounds described in Table I. Regression of the data on aliphatic derivatives of α-naphthol (filled circles) in A and B yield linear regression lines with correlation coefficients of 0.72 and 0.86, respectively. Aliphatic carbonates and thiocarbonates of α-naphthol and β-naphthol are represented by filled circles and squares, respectively, whereas aromatic analogues of α-naphthol are represented by open circles.

the trend of similar aliphatic derivatives (Fig. 2), it is suggested that some aspect of the mechanism of hydrolysis of these compounds differs from that of the aliphatic compounds. The most likely explanation for the relatively low hydrolytic rates of the aromatic analogues is a possible steric hindrance effect at the active site of the enzyme. Another possible explanation is the same argument presented for the alkyl carbonate series. This would then indicate, in addition to the naphthyl binding region, the presence of a fairly large hydrophobic region as part of, or in the neighborhood of, the active site in order to accommodate the bulky aryl carbonates. Increasing the polarizability of the carbonyl group of the aromatic analogues by introducing good leaving groups, such as *p*-nitrophenol in compound 8, did not lead to an increase in the turnover rates of these substrates.

The strong influence on esterase activities in rat liver and brain subcellular fractions and human plasma exerted by the lipophilicity nature of a series of nicotinate esters was also observed (29,30). It is clear that more detailed studies of the kinetics of the enzyme–substrate interacting are needed to reveal the true nature of the mechanism of hydrolysis of these carbonate derivatives.

Influence of pH on Mouse Liver Esterase Activities

The pH optimum for the hydrolysis of the acetate, methyl carbonate, and ethyl thiocarbonate of α-naphthol by murine liver esterase is shown in Fig. 3. Maximal esterase activity was observed from pH 7.0 to pH 7.6.

Table III. Kinetic Parameters of the Hydrolysis of Selected Substrates by Crude Mouse Liver Microsomes and Purified Hydrolases A and B from Rat Liver Microsomes[a]

Substrate no.	Enzyme source	V_{max} (μmol/min/mg protein)	K_m (μM)	V_{max}/K_m
1	Mouse	1.46 ± 0.05	50.1 ± 5.01	0.03
	Hydrolase A	17.7 ± 1.53	25.0 ± 5.10	0.71
	Hydrolase B	92.4 ± 4.48	48.1 ± 5.47	1.92
3	Mouse	ND[b]	ND	ND
	Hydrolase A	217 ± 25.2	199 ± 35.8	1.09
	Hydrolase B	115 ± 5.91	45.4 ± 5.55	2.54
5	Mouse	0.616 ± 0.046	6.99 ± 1.34	0.09
	Hydrolase A	4.45 ± 0.23	4.10 ± 0.76	1.09
	Hydrolase B	31.2 ± 1.41	16.6 ± 2.30	1.88
17	Mouse	0.291 ± 0.031	3.34 ± 1.10	0.09
	Hydrolase A	6.23 ± 0.38	4.35 ± 1.00	1.43
	Hydrolase B	ND	ND	ND
20	Mouse	9.11 ± 0.47	161 ± 16.7	0.06
	Hydrolase A	73.3 ± 3.70	129 ± 13.7	0.57
	Hydrolase B	248 ± 7.97	141 ± 9.41	1.77
21	Mouse	ND	ND	ND
	Hydrolase A	98.8 ± 2.77	81.5 ± 4.53	1.21
	Hydrolase B	395 ± 72.9	240 ± 66.2	1.65
22	Mouse	ND	ND	ND
	Hydrolase A	234 ± 19.3	204 ± 26.0	1.15
	Hydrolase B	282 ± 24.9	165 ± 24.0	1.71

[a] Assay conditions are described under Materials and Methods. Each value represents the means ± SD of three determinations.
[b] Not determined.

Hydrolysis of *p*-Nitrophenyl Derivatives

Of the *p*-nitrophenyl substrates tested, *p*-nitrophenyl acetate was cleaved most rapidly by human and mouse liver esterases (Table II). The enzymatic hydrolysis of *p*-nitrophenyl *N*-methyl carbamate could not be studied because of a vary rapid spontaneous hydrolysis.

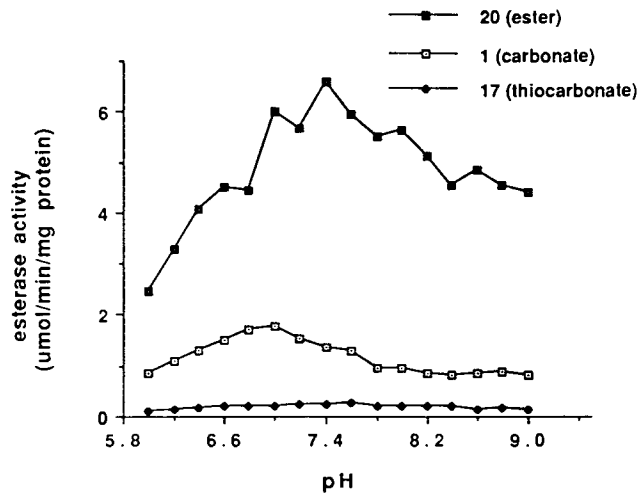


Fig. 3. Effect of pH on the hydrolysis of acetate, methyl carbonate, and ethyl thiocarbonate of α-naphthol by murine liver esterase. The data represent the mean of four determinations with the standard deviation for all points less than 5%. The optimum pH with ethyl-thiocarbonate of α-naphthol, 17, is about 7.6.

Chemical Hydrolysis of Selected α-Naphthyl Derivatives

The nonenzymatic hydrolytic rates of selected substrates in 0.1 *M* Tris–HCl buffer (pH 9.0) at 23°C indicates that the carbonates were generally more stable than the corresponding carboxylic esters (Table IV). Substrate 3, unlike the enzyme reaction, was hydrolyzed more rapidly than the model substrate, 20. This can be attributed to the specificity of the enzyme rather than the solubility of the substrate. Although the carbonate moiety of substrates 1 and 2 reduced the specific activities of the enzymes about fivefold compared to the corresponding carboxylates, it also reduced the spontaneous chemical hydrolysis significantly.

Table IV. Nonenzymatic Hydrolysis of Selected Substrates in 0.1 *M* Tris–HCl, pH 9.0, at 23°C[a]

Substrate no.	Derivative of α-naphthol	Apparent first-order rate constant (min^{-1})
1	Methyl carbonate	0.047
2	Ethyl carbonate	0.044
3	Vinyl carbonate	0.100
5	Isobutyl carbonate	0.028
17	Ethyl thiocarbonate	0.017
20	Acetate	0.073

[a] The final substrate concentrations were 0.5 m*M*. The apparent first-order rate constant was calculated by recording the absorbance of the reaction at 450 nm at regular intervals of time according to the assay procedure described under Materials and Methods.

Conclusion

Two main features of the substrate structure seem to influence the activity of the mammalian esterases: the type of functional group(s) and the lipophilicity of the molecules. The carboxylates of α- and β-naphthols with low lipophilicities (log$P$ < 4) and short acyl chains were hydrolyzed very rapidly by the mammalian esterases. α-Naphthyl carboxylates with log$P$ values > 4 were poorly hydrolyzed by these enzymes. Marked substrate selectivities were observed between rat liver hydrolase A and rat liver hydrolase B, with most of these compounds being better substrates for hydrolase B. The esterase activities of human and mouse liver microsomes were about five orders of magnitude smaller than that of rat hydrolase B. The relationship between the specific activities of the enzymes and the lipophilicity of the α- and β-naphthyl carbonate series substrates indicates that the enzymes showed decreasing activity with increasing lipophilicity of the substrates. Although the carbonate moiety of these substrates lowered the enzymatic hydrolytic rates compared to the carboxylates, it also improved the signal-to-noise ratio significantly under the assay conditions employed. This study supports the proposition that hydrophobic interactions play an integral part in the hydrolysis of esters and carbonates catalyzed by carboxylesterases, in terms both of binding of the substrates and of esterolytic activity. Polar or charged substrates are not preferred substrates for these enzymes. However, the effect of steric hindrance at the active site of the enzyme(s) caused by bulky substituents such as the aryl carbonates of α-naphthol may also influence the esterolytic activities of these enzymes.

ACKNOWLEDGMENTS

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