four protein subunits with different catalytic specificities explain the relationship between six basic glutathione *S*-transferases in rat liver cytosol. *J Biol Chem* **257**: 9909–9912, 1982.
7. Adams PA, Byfield MP, Goold RD and Thumser AE, The kinetics of heme-octapeptide (microperoxidase-8; MP-8) formation studied by HPLC monitoring of the peptic and tryptic hydrolysis of horse heart cytochrome-*c*. *J Inorg Biochem*, in press.
8. Habig WH and Jakoby WB, Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol* **77**: 398–405, 1981.
9. Ivanetich KM, Ziman MR, Mennie REM, Eidne KA, Corrigall A and Kirsch RE, Inhibition of rat GSH *S*-transferases by ethylene dibromide. *Res Commun Chem Pathol Pharmacol* **45**: 233–242, 1984.
10. Harvey JW and Beutler E, Binding of heme by glutathione *S*-transferase: a possible role of erythrocyte enzyme. *Blood* **60**: 1227–1230, 1982.
11. Corrigall AV, Ivanetich KM and Kirsch RE, Inhibition of human glutathione *S*-transferases by 1-chloro-2,4-dinitrobenzene. *Hepatology* **6**: 1136, 1986.

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Hydrolysis of phthalate esters by purified rat and human liver carboxylesterases

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Because of their wide use as plasticizers and production in millions of tons per year, phthalate esters have become environmental pollutants. Phthalates or metabolites of them are suspected to be hepatocarcinogenic [1, 2] and teratogenic [3] after chronic exposure and/or high dosage. Their metabolism in rats and humans involves the hydrolysis of one ester bond, and further metabolites are formed from the monoesters [1, 4]. Hydrolytic activity has been detected in rat pancreas, liver, mucosa, kidney and lung [5], but apart from the characterization of a pancreatic lipase [6] the identification of mammalian esterases or lipases involved in the metabolism of phthalate esters is unsatisfactory so far. Since in mammals hepatic carboxylesterases are mainly responsible [7, 8] for the hydrolysis of many ester- or amide-type drugs, we determined the action of purified carboxylesterases from rat and human liver on various phthalate diesters, in order to evaluate the contribution of these detoxication enzymes on the metabolism of these xenobiotics.

Materials and methods

Materials. Phthalate esters were purchased in highest obtainable purity from Fluka, or Merck (Darmstadt, F.R.G.) (dibutyl, diallyl, dicyclohexyl), except di(2-methoxyethyl), di(2-ethylhexyl) and diisobutyl phthalates which were generous gifts from Badische Anilin- und Sodafabrik, Ludwigshafen, F.R.G. The monobutyl ester was synthesized from equimolar amounts of phthalic acid anhydride and *n*-butanol in the presence of pyridine (2 hr, 95°). After acidification with hydrochloric acid, the monoester was purified by repeated crystallization from ethanol (m.p. of the product 74°).

Enzymes. Carboxylesterases were highly purified from rat [9] and human [10] liver microsomes as described earlier.

Enzyme assays. The hydrolysis of phenyl butyrate, methyl butyrate, 4-nitrophenyl acetate and palmitoyl-CoA was followed as described previously [11]. Phthalate esters were emulsified by ultrasonication (2 min, 150 W; Branson B 12 sonifier from Branson, Danbury, CT) immediately before assay in 0.1% Triton X-100 to yield 5 mM emulsions (or solutions). After registration of the blank with 5 ml of these substrate emulsions, the purified esterases in 100 μl 10 mM Tris–HCl buffer, pH 8.0, were added and the enzymatic hydrolysis was monitored at pH 8.0 and 37° by the pH-stat method using the autotitrator TTT 11 (Radiometer, Copenhagen, Denmark) with an automatic burette filled with 40 mM NaOH. One unit is defined as the enzymatic generation of one μmole carboxyl-groups per min. Protein was determined by the biuret procedure [9]. The possible enzymatic generation of phthalic acid was further investigated in some of the assays by spectrophotometric determination of the acid according to Takeuchi *et al.* [12].

Results and discussion

Three genetically distinct carboxylesterases (see Refs. 13 and 14 for classification and substrates), namely esterase pI 5.6 (ES-3, acetanilid hydrolase, lysophospholipase), esterase pI 6.0 (ES-8/ED-10, esterase E1, medium-chain monoacylglycerol lipase) and esterase pI 6.2/6.4 (ES-4, long-chain monoacylglycerol lipase) have been identified in rat liver endoplasmic reticulum as the most important xenobiotic-hydrolyzing carboxylesterases of this tissue. From human liver only one prominent detoxifying carboxylesterase is known [10, 15]. With common esterase substrates we measured for the highly purified esterases used in this study the following specific activities: with 1 mM phenyl butyrate at pH 8.0 and 37° rat esterase pI 5.6 192 U/mg, rat esterase pI 6.0 502 U/mg and rat esterase pI 6.2/6.4 140 U/mg; human esterase: 320 U/mg with 10 mM methyl butyrate at pH 8.0 and 30°, 101 U/mg with 0.5 mM 4-nitrophenyl acetate at pH 8.0 and 30°. These values are comparable or higher to activities reported earlier [10, 11].

The activities of these esterases on phthalate esters are summarized in Table 1. The diethyl, diallyl and dibutyl phthalates were generally the best esterase substrates, whereas strong hydrophobic diesters like diethylhexyl phthalate were not significantly hydrolyzed. Incorporation of other detergents as Triton X-100 in the assay (bovine serum albumin, taurocholate, no detergent) did not significantly moderate the activities of human or rat esterase pI 6.2/6.4 (determined with dibutyl phthalate as substrate). Monophthalate esters were not measurably hydrolyzed, and no free phthalic acid could be detected by the photometric method of Takeuchi *et al.* [12] even after prolonged incubation times (120 min, assays only with human esterase). It is therefore concluded that the liver esterases cleave only one ester bond from phthalate diesters. This is in accordance with the observation that charged esters like the monoesters are bad substrates for liver carboxylesterases [11, 15].

When the activities of the three genetically distinct rat liver esterases are compared to each other, the isoenzyme

pI 6.2/6.4 (ES-4) is the most active. This esterase is known to hydrolyze certain lipids (long-chain monoacylglycerides and CoA-esters, retinyl palmitate) and aromatic drugs (aspirin, propanidid) better than the other two isoenzymes [14, 16]. The activities of human liver esterase in hydrolyzing the phthalate esters were similar to those of the rat liver esterases, namely somewhat lower than those of esterase pI 6.2/6.4, but for the more lipophilic esters higher than those of the two other rat esterases. It should be noted that beside several drugs this human liver esterase cleaved also palmitoyl-CoA ($K_m = 28\,\mu M$, $V_{max} = 0.32$ U/mg) though with lower activity than the rat enzyme.

Some data about the influence of the structures of the phthalates on the kinetic constants measured for the human and most active rat esterase are presented in Table 2 and Fig. 1. The K_m-values decrease with increasing lipophilicities of the compounds hydrolyzed (Table 2), as found for other esterase substrates (R. Mentlein, unpublished) or other hydrolytic enzymes [17]. According to Fig. 1 the reaction rates for the enzymatic hydrolysis show a maximum for a certain lipophilicity of the diesters, i.e. diallylphthalate. Lower as well as higher lipophilicities of the phthalates yield lower reaction rates especially in the case of the most active rat liver esterase. Lipophilicity data were taken from Leyder and Boulanger [18] or calculated from the correlation between the lipophilicities of the diesters and the corresponding alcohols: log $P_{diester} = 1.80$ (± 0.12) log $P_{alcohol}$ + 2.90 (± 0.08), $r = 0.992$, N = 5 (data from Leyder and Boulanger [18] and Hansch and Leo [19]). However, structure–activity relationships are more complex and may differ between different esterases, e.g. the human enzyme hydrolyzed the ester of the secondary alcohol isobutanol more slowly than the corresponding primary one, whereas the rat liver esterase of pI 6.2/6.4 hydrolyzed diisobutyl phthalate more quickly than di-*n*-butyl phthalate.

The optimal cleavage rate for compounds of moderate lipophilicities distinguishes liver esterases from the pancreatic lipase investigated by Albro and Thomas [5]. The latter enzyme is also active on highly lipophilic compounds like diethylhexyl phthalate. Since the carboxylesterases investigated here contribute significantly to the hydrolytic activity of the liver, the esterases are most likely participating in the metabolism of these environmental pollutants also *in vivo*.

Table 1. Hydrolysis of phthalate esters by purified rat and human liver carboxylesterases

Phthalate ester	Specific activity (μmol/min/mg protein) determined with purified human esterase	rat esterase pI 5.6	rat esterase pI 6.0	rat esterase pI 6.2/6.4
Monomethyl	<0.1	<0.1	<0.1	<0.1
Monobutyl	<0.1	<0.1	<0.1	<0.1
Dimethyl	4.1	4.2	3.2	3.9
Diethyl	4.7	3.9	14.9	14.1
Diallyl	5.2			16.7
Di(2-methoxyethyl)	1.2			4.2
Dibutyl	3.2	1.7	0.5	4.1
Diisobutyl	1.2	0.7		7.4
Dibenzyl	1.2			1.2

Values were determined with 5 mM emulsions/solutions of the esters at pH 8.0 and 37°.

No significant activities (<0.5) were measured with di-(2-ethyl)hexyl, di-(3.5.5′-trimethyl)-hexyl, dinonyl, diisononyl, diisodecyl, diisotridecyl and dicyclohexyl phthalates.

Table 2. Some kinetic constants determined for the hydrolysis of phthalate esters by purified rat liver carboxylesterases

Phthalate ester	log *P*	Esterase pI 5.6 K_m (mM)	Esterase pI 5.6 V_{max} (μmol/min/mg)	Esterase pI 6.2/.64 K_m (mM)	Esterase pI 6.2/.64 V_{max} (μmol/min/mg)
Dimethyl	1.53	0.95	4.96	1.92	5.41
Diethyl	2.35	0.78	4.54	1.33	17.82
Dibutyl	4.57	0.15	1.72	0.15	4.20
Diisobutyl	4.11	0.15	0.76	0.19	7.68

Enzymatic hydrolysis was determined by autotitration at pH 8.0 and 37% with approx. 100 μg esterase and various concentrations of the esters between 0.2 and 5 mM. The log *P* values are taken from Ref. 18 and were determined experimentally with octanol–water at 20°

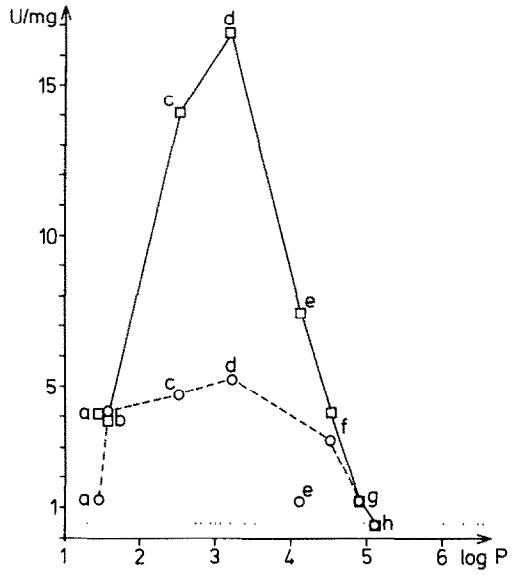

Fig. 1. Lipophilicities of phthalate diesters plotted vs the enzymatic hydrolysis rates determined with human (○--○) and rat pI 6.2/6.4 (△—△) liver carboxylesterases. Dotted line: detection limit for enzymatic hydrolysis. Phthalate esters: a, di-(2-methoxy-ethyl); b, dimethyl; c, diethyl; d, diallyl; e, diisobutyl; f, dibutyl; g, dibenzyl; h, dicyclohexyl.

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* *Anatomisches Institut*
Universität Kiel
Olshausenstrasse 40
D-2300 Kiel
Federal Republic of Germany
‡ *FB Chemie*
Universität Oldenburg
Ammerländer Heerstrasse 114
D-2900 Oldenburg
Federal Republic Germany

ROLF MENTLEIN*†
WERNER BUTTE‡

REFERENCES

1. Ganning AE, Brunk U and Dallner G, Phthalate esters and their effect on the liver. *Hepatology* **4**: 541–547, 1984.
2. Kluwe WM, McConnell EE, Huff JE, Haseman JK, Douglas JF and Hartwell WV, Carcinogenicity testing of phthalate esters and related compounds by the national toxicology program and the national cancer institute. *Environ Health Perspec* **45**: 129–133, 1982.
3. Shiota K and Nishimura H, Teratogenicity of di(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) in mice. *Environ Health Perspec* **45**: 65–70, 1982.
4. Thomas JA and Northup SJ, Toxicity and metabolism of monoethylhexyl phthalate and diethylhexyl phthalate: a survey of recent literature. *J Toxicol Environ Health* **9**: 141–152, 1982.
5. Albro PW and Thomas RO, Enzymatic hydrolysis of di-(2-ethylhexyl) phthalate by lipases. *Biochim Biophys Acta* **360**: 380–390, 1973.
6. Albro PW, Corbett BJ and Latimer AD, Purification and partial characterization of nonspecific lipase from rat pancreas. *Biochim Biophys Acta* **424**: 351–365, 1976.
7. Heymann E, Hydrolysis of carboxylic esters and amides. In: *Metabolic Basis of Detoxication* (Ed. Jacoby WB), pp. 229–245. Academic Press, New York, 1982.
8. Leinweber F-J, Possible physiological roles of carboxylic ester hydrolases. *Drug Metabolism Rev* **18**: 379–439, 1987.
9. Mentlein R, Heiland S and Heymann E, Simultaneous purification and comparative characterization of six serine hydrolases from rat liver microsomes. *Arch Biochem Biophys* **200**: 547–559, 1980.
10. Junge W, Heymann E and Krisch K, Human liver carboxylesterase, purification and molecular properties. *Arch Biochem Biophys* **165**: 749–763, 1974.
11. Mentlein R, Suttorp M and Heymann E, Specificity of purified monoacylglycerol lipase, palmitoyl-CoA hydrolase palmitoyl-carnitine hydrolase, and nonspecific carboxylesterase from rat liver microsomes. *Arch Biochem Biophys* **228**: 230–246, 1984.
12. Takeuchi T, Horikawa R and Tanimura T, Spectrophotometric determination of carboxylic acids by ferric hydroxamate formation with water-soluble carbodiimide. *Analyt Lett* **13**: 603–609, 1980.
13. Mentlein R, Ronai A, Robbi M, Heymann E and von Deimling O, Genetic identification of rat liver carboxylesterases isolated in different laboratories. *Biochim Biophys Acta* **913**: 27–38, 1987.
14. Heymann E and Mentlein R, Identification, localization and specificity of detoxifying hydrolases in rat tissues. In: *Metabolism of Xenobiotics* (Eds. Gorrod JW, Oelschläger H and Caldwell J), pp. 189–197. Taylor & Francis, London, 1988.
15. Heymann E, Carboxylesterases and amidases. In: *Enzymatic Basis of Detoxication, Vol. II* (Ed. Jakoby WB), pp. 291–324. Academic Press, New York, 1980.
16. Mentlein R and Heymann E, Hydrolysis of ester- and amide-type drugs by the purified isoenzymes of nonspecific carboxylesterase from rat liver. *Biochem Pharmacol* **33**: 1243–1248, 1984.
17. Järv J, Kesvatera T and Aaviksaar A, Structure–activity relationships in acetylcholinesterase reactions. *Eur J Biochem* **67**: 315–322, 1976.
18. Leyder F and Boulanger P, Ultraviolet absorption, aqueous solubility, and octanol-water partition of several phthalates. *Bull Environ Contam Toxicol* **30**: 152–157, 1983.
19. Hansch C and Leo A, *Substituent Constants for Correlation Analysis in Chemistry and Biology*. Wiley-Interscience, New York, Chichester, 1979.

† To whom correspondence should be addressed.