Biochemical Pharmacology, Vol. 29, pp. 1927–1931.

0006-2952/80/0701-1927 $02.00/0

SELECTIVE INHIBITION OF RAT LIVER CARBOXYLESTERASES BY VARIOUS ORGANOPHOSPHORUS DIESTERS *IN VIVO* AND *IN VITRO*

ERNST BRANDT, EBERHARD HEYMANN and ROLF MENTLEIN

Biochemisches Institut, Fakultät Medizin, Universität Kiel, Olshausenstraße, D-2300 Kiel, Federal Republic of Germany

(*Received* 17 *December* 1979; *accepted* 28 *February* 1980)

Abstract—Five purified carboxylesterases/amidases of rat liver are susceptible to inhibition by various organophosphorus diesters and triesters. The inhibitory effect of these esters generally decreases in the order paraoxon>bis(4-nitrophenyl)phosphate>4-nitrophenyl-phenyl-phosphate>bis(4-cyanophenyl)phosphate>2-nitrophenyl-phenyl-phosphate. After *in vivo* application of radioactive bis(4-nitrophenyl)phosphate, all microsomal carboxylesterases of rat liver—including the five hydrolases investigated *in vitro*—are irreversibly labeled. We conclude that all carboxylic ester hydrolases of rat liver that act at neutral pH can be classified as serine hydrolases. The hydrolases pI 6.2 and 6.4 that have the highest monoleylglycerol-cleaving activities are relatively insensitive to inhibition by organophosphorus diesters as compared to the hydrolases with lower pI. The very similar hydrolases pI 6.2 and 6.4 may be distinguished *in vitro* by the action of 0.1 mM $HgCl_2$, which inhibits hydrolase pI 6.4 almost completely. Among the organophosphorus diesters investigated, bis (4-cyanophenyl)phosphate is a surprisingly specific inhibitor for the acetanilide-cleaving hydrolase pI 5.6 both *in vitro* and *in vivo*. This inhibitor allows a clear discrimination between the hydrolases pI 5.2 and 5.6 that have similar substrate specificity and chemical properties. After intraperitoneal injection into mice, bis(4-cyanophenyl)phosphate exhibits extremely low toxicity.

Recently, we described the isolation of six serine hydrolases from rat liver [1]. Individual members of this group of closely related enzymes have previously been classified as carboxylesterases (EC 3.1.1.1.), aryl acylamidases (EC 3.5.1.13) and monoacylglycerol lipases (EC 3.1.1.23) [1]. Since all of the purified hydrolases split amides, monooleylglycerol and simple aliphatic and aromatic esters (see ref. 1 and unpublished results), and unambiguous classification is not possible at present. Therefore, we use here the preliminary nomenclature introduced in our preceding paper [1] (compare ref. 2). Although a physiological function of this group of enzymes is not known, it is obvious that they play an important role in drug metabolism [2].

A commonly used scheme for the classification of carboxylesterases and related enzymes (A-, B- and C-esterases) is based on their behaviour towards certain inhibitors [2], especially organophosphates and mercury compounds. The objectives of this investigation were to find out whether this scheme can be applied to the five isolated enzymes, and to detect inhibitors that allow differentiation between the rat liver isoenzymes.

Bis(4-nitrophenyl)phosphate is a well known inhibitor of liver carboxylesterases [3]. It acts by irreversible phosphorylation of the active sites of these enzymes and it has been widely used both in biochemical [1, 2, 4–6] and in pharmacological studies [2, 7–9]. A further objective of this study is to decide which of the many carboxylesterase isoenzymes of rat liver are susceptible to inhibition by bis(4-nitrophenyl)phosphate and related organophosphorus diesters.

MATERIALS AND METHODS

Enzymes and reagents

Rat liver microsomes and the five highly purified microsomal hydrolases were prepared as previously described. Paraoxon (diethyl-4-nitrophenyl-phosphoric acid triester) was a gift from Bayer AG (Leverkusen, F.R.G.). Bis(4-nitro[^{14}C]phenyl)phosphate was from Hoechst AG (Frankfurt, F.R.G.). Unlabeled bis(4-nitrophenyl)phosphate was from Merck (Darmstadt, F.R.G.), 4-hydroxy-mercuribenzoate (former name: *p*-chloro-mercuribenzoate) was from Serva, Heidelberg, F.R.G. Bis(4-cyanophenyl)-phosphate (cyclohexylammonium salt) was synthesized according to Kolbe [10]. Thanks are due to Drs. Kolbe and Schnekenburger (Kiel, F.R.G) for the gift of 4-nitrophenyl-phenyl-phosphoric acid diester and 2-nitrophenyl-phenyl-phosphoric acid diester (cyclohexyl-ammonium salts).

Enzyme assays

The hydrolysis of methyl butyrate (20 mM) was followed titrimetrically at pH 8.0 and 30° by means of the pH-stat technique (autotitrator TTT 1c with automatic burette and continuous registration of the NaOH consumption, Radiometer, Copenhagen, Denmark).

The liberation of nitrophenol from 4-nitrophenylacetate (1.6 mM) at pH 8.0 and 30° was followed spectrophotometrically [11]. The enzymatically-formed aniline from acetanilide (10 mM) at pH 8.6 and 30° was determined by a modification of the diazotization procedure of Brodie and Axelrod [12]. The enzyme concentrations for the esterase tests

were in the range of 1 μg/ml, and for the amidase test in the range of 10 μg/ml.

In vitro *inhibition experiments*

Samples of approximately 20 μg purified hydrolases or 1 mg of microsomal protein were preincubated with the inhibitor in the absence of substrate (total volume: 0.1 ml of 20 mM phosphate buffer, pH 8.0, 30°). Aliquots of 10 or 50 μl were taken from the mixture and analysed for remaining esterase or amidase activity. The inhibition was referred to parallel controls without inhibitors.

Application of phosphoric acid diesters in vivo. Adult female Wistar rats weighing about 200 g received intraperitoneal (i.p.) injections of bis-(4-nitro[^{14}C]phenyl)phosphate (specific radio activity 75 μCi/mg; dose 10 mg/kg) or of bis-(4-cyanophenyl)phosphate (100 mg/kg) dissolved in 0.9% saline. A control series received an equal volume of saline without inhibitor. The animals were decapitated and liver microsomes were prepared by differential centrifugation [13].

The toxicity of bis(4-cyano-phenyl)phosphate was assayed in several groups of ten female mice of the NMRI strain (Hagemann, Bösingfeld, F.R.G.) that received i.p. injections of this inhibitor in saline. The highest concentrations (700 mg/kg) were injected as suspensions dispersed by ultrasonification. Twenty-four hours after these injections the animals were decapitated and the carboxylesterase/amidase activities of the liver microsomes were assayed as described above.

Isoelectric focusing

Analytical isoelectric focusing in polyacrylamide slab gels was performed as described earlier [1]. The microsomal samples contained 1% Nonidet P 40 to solubilize the esterases and amidases [1]. The gels were stained for esterase activity with 1-naphthyl-acetate [1]. After staining (3–4 min), the protein bands were precipitated in the gels by incubation with 12.5% trichloroacetic acid [1 hr]. The gels were then washed with 7% acetic acid (6 hr) and dried on filter paper *in vacuo*. For autoradiography the dried gels were pressed onto X-ray films (Agfa–Gevaert Osray T 4) for several weeks.

RESULTS AND DISCUSSION

Influence of various inhibitors on purified carboxylesterase/amidase isoenzymes in vitro. The five carboxylesterases/amidases investigated here represent almost the entire activity of rat liver against simple aromatic and aliphatic esters at neutral and slightly alkaline pH [1]. The results of our *in vitro* inhibition experiments are summarized in Table 1. The complete inhibition of all carboxylesterase/amidase isoenzymes by paraoxon confirms that they are serinehydrolases [1]. 4-Hydroxymercuribenzoate is practically without effect; thus these enzymes bear no SH-groups that are essential for their activity. This observation is contrary to an earlier report on the hydrolase pI 5.6 [14]. No mercury-activable enzyme (C-esterase [2]) is among the hydrolases investigated here. In contrast, $HgCl_2$ is a powerful inhibitor of all isoenzymes. The inhibition is practically complete with the hydrolase pI 6.4. We conclude from these results that no A- or C-esterases are among the five enzymes investigated here, and that the five esterases can unambiguously be classified as B-esterases according to the scheme introduced by Aldridge [15].

It can be assumed that the inhibitory efficiency of all phosphoric acid diesters is based on an irreversible phosphorylation of the active site of liver carboxylesterases/amidases, as has been shown for bis(4-nitrophenyl)phosphate [16]. If this is so, the figures in Table 1 correspond to differing phosphorylation velocities: Low values of remaining activity correspond to high rates and vice versa. The rate of inhibition by organophosphorus esters for all isoenzymes decreases in the order paraoxon > bis-(4-nitrophenyl)phosphate > (4-nitrophenyl)phenyl-phosphate>(2-nitrophenyl)phenyl phosphate. The effect of bis(4-cyanophenyl)phosphate is similar to that of the last compound, except for its action on hydrolase pI 5.6. The surprisingly specific inhibition of this acetanilide-cleaving enzyme by bis(4-cyanophenyl)phosphate is confirmed by the corresponding experiment with microsomes (Table 1), where only the acetanilide-cleaving activity is completely inhibited, and by the *in vivo* studies (see below). This result is relevant to drug metabolism, because the hydrolase pI 5.6 is responsible for the hydrolysis of procaine and phenacetin in liver (R. Mentlein and E. Heymann, unpublished results).

Among the five hydrolases investigated, those with

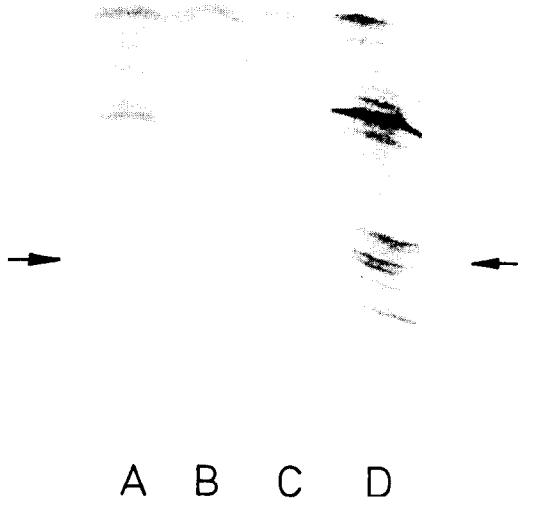

Fig. 1. Isoelectric focusing of extracts from rat liver microsomes after injection of inhibitors *in vivo*. Liver microsomes obtained after decapitation of the animals were treated with 1% Nonidet P 40. A: Untreated rat; esterase stain. B: Rat killed 3 hr after i.p. injection of bis(4-cyanophenyl)phosphate (100 mg/kg); esterase stain. C: Rat killed 3 hr after i.p. injection of 10 mg bis(4-nitro[^{14}C]phenyl)phosphate per kg body wt; esterase stain. D: Autoradiograph of C.

Table 1. Effect of inhibitors on rat liver hydrolases *in vitro*

Enzyme	Substrate	Remaining activity* (%)						
					Phosphoric acid diesters			
		$HgCl_2$	4-Hydroxy-mercuri-benzoate	Paraoxon	$(R^1O)_2PO_2^-$	$(R^1O)(R^2O)PO_2^-$	$(R^2O)(R^3O)PO_2^-$	$(R^4O)_2PO_2^-$
Hydrolase (pI 5.2)	4-Nitrophenyl acetate	30	92	0	11	42	61	76
	Methyl butyrate	26	86	0	3	35	58	82
Hydrolase (pI 5.6)	4-Nitrophenyl acetate	17	95	0	13	35	49	6
	Methyl butyrate	16	101	0	2	25	40	0
	Acetanilide	31	99	0	4	31	44	3
Hydrolase (pI 6.0)	4-Nitrophenyl acetate	29	95	0	4	52	73	64
	Methyl butyrate	24	111	0	6	57	70	68
Hydrolase (pI 6.2)	4-Nitrophenyl acetate	30	98	0	33	92	100	97
Hydrolase (pI 6.4)	4-Nitrophenyl acetate	2	100	0	56	67	100	100
Microsomes	4-Nitrophenyl acetate	28	95	0	20	45	89	63
	Methyl butyrate	30	104	0	12	40	47	22
	Acetanilide	45	104	0	0	36	44	0

* Enzyme was preincubated with inhibitor (0.1 mM) for 30 min (30°, pH 8.0); results are mean values of three experiments. R^1 = 4-nitrophenyl, R^2 = phenyl, R^3 = 2-nitrophenyl, R^4 = 4-cyanophenyl.

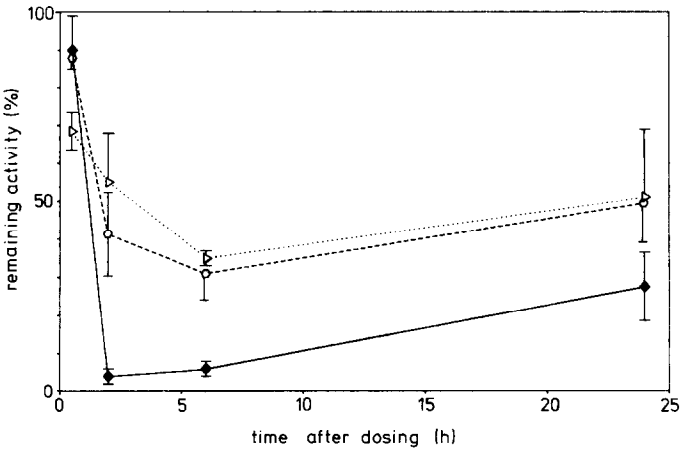

Fig. 2. Kinetics of esterase/amidase inhibition and recovery after i.p. injection of bis(4-cyanophenyl)phosphate in rats. A single dose of bis(4-cyanophenyl)phosphate (100 mg/kg) in 0.9% saline was injected into female rats. The animals were decapitated at varying times after the injection and the enzyme activity was estimated in the liver microsomes. Enzyme activities are given in per cent of the control group that received a single injection of saline without inhibitor. Each symbol represents the mean of six animals ± S.D. Hydrolysis of ▷···▷ methyl butyrate, ○--○ 4-nitrophenyl acetate and ◆—◆ acetanilide.

pI 6.2 and 6.4 are relatively insensitive to inhibition by the organophosphorus diesters (Table 1). However, since all of the isoenzymes bind bis(4-nitrophenyl)phosphate irreversibly (Fig. 1), it can still be assumed that they are susceptible to phosphorylation by these esters.

In vivo *effects of bis (4-cyanophenyl)phosphate.* After i.p. injection of bis(4-cyanophenyl)phosphate in rats (100 mg/kg), only the multiple forms of the acetanilide-cleaving esterase (arrow in Fig. 1) are inhibited completely (see l.c. [1] for the correlation of the isoelectrofocusing band pattern with the purified esterases).

On the other hand, the structurally very similar inhibitor bis(4-nitrophenyl)phosphate is bound to all of the esterase isoenzymes (l.c. [16], compare fig. 1D), though the inhibition is incomplete especially with the higher pI isoenzymes. Thesefore, it can be assumed that bis(4-cyanophenyl)phosphate also binds to all isoenzymes. This assumption has to be confirmed by an experiment with radioactive inhibitor which is not yet available.

Figure 2 shows the kinetics of esterase/amidase inhibition and recovery after i.p. injection of bis(4-cyanophenyl) phosphate into adult female Wistar rats. Only the acetanilide-cleaving activity is almost completely inhibited 2 hr after the injection. This agrees well with the *in vitro* results, because this activity can almost entirely be attributed to the hydrolase pI 5.6. In contrast, bis(4-nitrophenyl)phosphate inhibits esterolytic activities and the acetanilide-cleaving activity at very similar rates [9]. The kinetics shown in Fig. 2 differ from similar experiments with bis(4-nitrophenyl)phosphate [9] insofar as the inhibition is generally slower with the new inhibitor. The recovery of enzyme activity can be attributed to the *de novo* synthesis of these enzymes [16]. In female mice the difference in the degree of inhibition of the acetanilide- and the ester-cleaving activities after i.p. injection of bis(4-cyanophenyl) phosphate (100 mg/kg) is less pronounced than in rats. Twenty-four hours after application of the inhibitor the remaining liver activities were 11.2 per cent for methyl butyrate, 16.8 per cent for 4-nitrophenyl acetate and 18.3 per cent for acetanilide (mean values obtained from three animals; control group without injection = 100 per cent). It is remarkable that after injection of only 10 mg/kg the remaining activities were almost the same: 11.7 per cent for methyl butyrate, 17.3 per cent for 4-nitrophenyl acetate and 19.3 per cent for acetanilide (N = 3). This suggests a high affinity of bis(4-cyanophenyl)phosphate to mouse liver esterases/amidases, but unlike rats no preferential inhibition of the amidase activity is found. From published data [9, 16] on the *in vivo* action of bis(4-nitrophenyl)phosphate on rat liver carboxylesterases/amidases, it can be concluded that the action of this inhibitor is similarly independent of the applied dose: i.p. injection of both 10 and 100 mg/kg leads to about 90% inhibition of the liver carboxylesterases.

The toxicity of bis(4-cyanophenyl)phosphate seems to be very low. It was not possible to kill mice by i.p. injections of 700 mg of this diester per kg body wt. The LD_{50} of bis(4-nitrophenyl)phosphate has been reported to be 410 mg/kg in mice [3]. However, this difference might be due to the toxicity of 4-nitrophenol that is cleaved from bis(4-nitrophenyl)phosphate by phosphodiesterases [17]. Rats survive intraperitoneal injections of 100 mg bis(4-nitrophenyl)phosphate or bis(4-cyanophenyl)-phosphate per kg body wt without impairment of their well-being.

Acknowledgements—The skilful technical assistance of Mrs. H. Rix and Mrs. C. Szeiki is gratefully acknowledged. We thank Dr. W. Kolbe and Dr. J. Schnekenburger for their gift of organophosphorus diesters. Thanks for stylistic advice are due to Dr. A. Corfield.

REFERENCES

1. R. Mentlein, S. Heiland and E. Heymann, *Archs. Biochem. Biophys.* **200**, in press.
2. E. Heymann, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby), *Monographs in Biochemical Pharmacology and Toxicology*, Vol. 1. Academic Press, New York, in press.
3. E. Heymann and K. Krisch, *Hoppe-Seyler's Z. physiol. Chem.* **348**, 609 (1967).
4. M. Kunert and E. Heymann, *Fedr. Eur. biochem. Soc.* **49**, 292 (1975).
5. W. Block and R. Arndt, *Biochim. biophys. Acta* **524**, 85 (1978).
6. O. von Deimling and A. Boecking, *Histochem. J.* **8**, 215 (1976).
7. F. R. Murphy, V. Krupa and G. S. Marks, *Biochem. Pharmac.* **24**, 883 (1975).
8. N. Kolassa, E. Tuisl and O. Kraupp, *Biochem. Pharmac.* **27**, 2269 (1978).
9. E. Heymann, K. Krisch, H. Buech and W. Buzello, *Biochem. Pharmac.* **18**, 801 (1969).
10. W. Kolbe, Dissertation. University of Kiel, F.R.G. (1975).
11. K. Krisch, *Biochim. biophys. Acta* **122**, 265 (1966).
12. K. Krisch, *Biochem. Z.* **337**, 531 (1963).
13. E. Heymann, W. Junge, K. Krisch and G. Marcussen-Wulff, *Hoppe-Seyler's Z. physiol. Chem.* **355**, 155 (1974).
14. R. Arndt, H. E. Schlaak, D. Uschtrin, D. Südi, K. Michelssen and W. Junge, *Hoppe-Seyler's Z. physiol. Chem.* **359**, 641 (1978).
15. W. N. Aldridge, *Biochem. J.* **53**, 110 (1953).
16. E. Heymann, R. Mentlein, R. Schmalz, C. Schwabe and F. Wagenmann, *Eur. J. Biochem.* **102**, 509 (1979).
17. E. Brandt and E. Heymann, *Biochem. Pharmac.* **27**, 733 (1978).