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CARBOXYLESTERASES IN GUINEA-PIG PLASMA AND LIVER

TISSUE SPECIFIC REACTIVATION BY DIACETYLMONOXIME AFTER SOMAN INHIBITION *IN VITRO*

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Abstract—The carboxylesterase activity in both plasma and liver of guinea-pig were separated into three main peaks by chromatofocusing. Two of the three plasma enzymes were retained by affinity chromatography on Affi-Gel Blue (100–200 mesh). Isoelectric points determined by chromatofocusing or isoelectrofocusing were pI 6.1, pI 5.2 and pI 4.0 for the plasma enzymes, and pI 5.7, pI 5.2 and pI 4.5 for the liver enzymes. The effect of selective esterase inhibitors, soman, physostigmine (cholinesterase inhibitor) and bis-4-nitrophenyl phosphate (carboxylesterase inhibitor), suggested that the three enzymes in both tissues may be regarded as carboxylesterases. However, the pI 5.7 carboxylesterase was partially inhibited by physostigmine, and the pI 4.5 carboxylesterase was almost not affected by bis-4-nitrophenyl phosphate. The ratio between the activities towards 4-nitrophenyl butyrate and methyl butyrate differed among the carboxylesterases in both tissues. All three carboxylesterases in plasma were partially reactivated by diacetylmonoxime after soman inhibition *in vitro*, but to a different extent. The soman inhibited liver carboxylesterases were not reactivated by diacetylmonoxime.

Previous studies have demonstrated a scavenger function of carboxylesterase (EC 3.1.1.1) in the detoxification of the organophosphorus anticholinesterase soman in rodents [1–8]. Based on the fact that organophosphorus compounds bind covalently to the active site of this enzyme [9, 10], it was shown that especially the carboxylesterase in rodent plasma may be highly important as a scavenger for soman [2–8]. Carboxylesterase is detected in many tissues in multiple forms with different characteristics (see refs 11–13 for review). We have separated the carboxylesterase from different tissues into two main groups based on their activity towards aliphatic and aromatic butyric ester [14]. Recently we have also seen that the total carboxylesterase activity in rodent plasma and lung could be partly reactivated by diacetylmonoxime after soman inhibition, whereas commercial carboxylesterase preparation from porcine liver could not be reactivated [8].

Previously for guinea-pig liver the carboxylesterases were divided into two main groups based on their substrate specificity towards aliphatic ester and amides [15]. In serum at least two carboxylesterases were separated based on isoelectric focusing [16]. In the present study three carboxylesterases in both plasma and liver could be separated by chromatofocusing and partially by affinity chromatography and isoelectrofocusing. The different enzyme activities were characterized by their substrate specificity, specific inhibitors and by their reactivatability by diacetylmonoxime after soman inhibition.

MATERIALS AND METHODS

Chemicals

Methyl butyrate (purity: reference substance for gas chromatography min 99.5%), saponin (both from E. Merck AG), 4-nitrophenyl butyrate, bis-4-nitrophenyl phosphate sodium salt, physostigmine salisylate, imidazole (all from Sigma Chemical Co), diacetylmonoxime, glycylglycine, acetylcholine iodide (all from Fluka AG), Affi-Gel Blue 100–200 mesh (Bio-Rad), Ultrodex gel, pH 2.5–4 Ampholine (both from LKB), glycerol (Bethesda Research Lab.), Polybuffer, PBE 94, 3–10 Pharmalyte (all from Pharmacia) and [1-^{14}C]acetylcholine chloride (55 Ci/mole) (Amersham) were purchased. Methyl[1-^{14}C]butyrate (7.4 mCi/mole) was synthesized as previously described [14]. Soman (1,2,2-trimethylpropyl methylphosphonofluoridate) was synthesized in our laboratory and the purity found to be better than 97% by nuclear magnetic resonance spectroscopy.

Animals

Guinea pigs, female albino (300–390 g weight), were purchased from National Institute of Public Health, Oslo.

Preparation of tissues

Guinea pigs were anaesthetized with ether, and plasma and liver were collected as described [14]. Liver homogenate (10% w/v) in 0.02 M sodium phosphate buffer pH 7.4 was centrifuged (1100 *g*, 10 min), and 9 vol. of the supernatant was mixed

with 1 vol. of 10% saponin in the same buffer. After 1 hr the mixture was centrifuged (4300 *g*, 20 min) and the supernatant collected. Before chromatofocusing, plasma and liver supernatant were gelfiltrated on prepacked Sephadex G-25 columns (PD-10, Pharmacia) equilibrated and eluted with 0.025 M imidazole × HCL buffer pH 7.4. To each column was applied 3 ml of a 2-fold dilution of plasma in the same buffer or 3 ml of liver supernatant. The elution volume was 3.2 ml for each column, and a total of 6 ml eluate constituted the sample for chromatofocusing.

Before electrofocusing, plasma was chromatographed on Affi-Gel blue (100–200 mesh) at 5° to remove albumin, in principle as described by Cain *et al.* [16]. A column (2.6 × 27 cm) was packed with the gel and equilibrated with 650 ml of 0.1 M ammonium formate pH 8.0. A volume of 10 ml plasma was applicated, and the column was eluted with 240 ml of 0.1 ammonium formate pH 8.0. Elution rate was 10 ml/hr and 5 ml fractions were collected. After enzyme measurement, one half of each of 7 active fractions eluted at between 130 ml and 165 ml of buffer, were pooled and the protein was precipitated (>90% by OD_{280} measurement) by 80% ammonium sulphate saturation in 1 hr. The pellet after centrifugation (10,000 *g*, 10 min) was dissolved in 6 ml bidistilled water, and desalted on PD-10 columns equilibrated and eluted with bidistilled water. A total volume of 6 ml eluate constituted the sample for isoelectrofocusing.

Chromatofocusing

Plasma and liver preparations were chromatofocused at 5° mostly as described in chromatofocusing kit instructions (Pharmacia). The start buffer was 0.025 M imidazole × HCl pH 7.4. The elution buffer was an 8-fold dilution of Polybuffer × HCl pH 4. A column (0.9 × 24 cm) was packed with PBE 94 and equilibrated with 600 ml of start buffer. The sample (6 ml) was applicated by first running on 5 ml of the elution buffer. The elution was performed with 170–190 ml of elution buffer followed by 2 column volumes of 1 M NaCl solution. The elution rate was 18 ml/hr (plasma) or 36 ml/hr (liver), and 1.5 ml or 3 ml fractions were collected. The column was reused following equilibration with start buffer as above.

Isoelectrofocusing

After affinity chromatography and desalting, the eluate containing plasma carboxylesterase was electrofocused on granulated gel. The procedure was mostly as described by Cain *et al.* [16] in accordance with the LKB Application Note 198. The gel slurry (100 ml) contained 2% (w/v) ampholytes (1 vol. of pH 2.5–4 Ampholine plus 3 vol of 3–10 Pharmalyte), 10% (v/v) glycerol, 4% (w/v) Ultrodex gel and sample in bidistillated water. The gel was dried (36–38% evaporation), and electrophoresed at 8 W (constant power) for 17 hr with water cooling. After electrofocusing, each of 36 gel slices were eluted with 2 ml of 10% (v/v) glycerol, and the pH of the eluates was measured. Then to each fraction was added 0.2 ml of 0.1 M sodium phosphate buffer pH 7.4 before enzyme measurement.

Measurement of enzyme activities

Carboxylesterase. Carboxylesterase activity was measured spectrophotometrically with 4-nitrophenyl butyrate, and radiochemically with methyl-[1-^{14}C]-butyrate as the substrates, as previously described [14].

Cholinesterase. The hydrolysis of acetylcholine was measured by the radiochemical method of Sterri and Fonnum [17].

Reactivation studies

Reactivation studies were performed with the most active fraction of the carboxylesterase peaks separated by chromatofocusing of plasma and liver preparations. The enzyme solution was diluted 2-fold in 0.05 M (final conc.) glycyglycine buffer pH 10.1, and 9 vol. was incubated at 30° for 30 min with 1 vol. of 10 μM soman in water. At this pH excess of soman should be hydrolyzed during the incubation [18], which was confirmed in control experiments by addition of fresh enzyme after 30 min. After the incubation with soman, one vol of inhibited enzyme solution was mixed with 9 vol. of 1 mM diacetylmonoxime in 0.1 M sodium phosphate buffer pH 7.8. The mixture was incubated at 30°, and the hydrolysis of 4-nitrophenyl butyrate was measured after 5, 15 and 30 min. Control experiments were performed similarly omitting soman and/or diacetylmonoxime. The percentage of reactivation obtained was calculated according to de Jong and Wolring [18]:

$$\% \text{ reactivation} = \frac{\text{EIR(E/ER)} - \text{EI}}{\text{E} - \text{EI}} \cdot 100$$

where EIR is the activity restored by oxime after soman inhibition, E the activity omitting soman and oxime, ER the activity omitting soman, EI the activity omitting oxime.

RESULTS

Separation of plasma carboxylesterases

Both chromatofocusing as well as affinity chromatography followed by electrofocusing were used to separate the carboxylesterases in plasma. By chromatofocusing of the plasma preparation, two main peaks of carboxylesterase activity could be detected after elution with the Polybuffer and a third peak, also containing cholinesterase activity, was detected after elution with NaCl-solution (Fig. 1). The ratio between the activities towards 4-nitrophenyl butyrate and methyl butyrate were quite similar for the most active fraction of the two peaks eluted by Polybuffer, and distinctly different from that of the third peak (Fig. 1, Table 1). The same fractions were also tested for the effect of selective esterase inhibitors on 4-nitrophenyl butyrate hydrolysis. The results showed that all three activity peaks were inhibited by soman and bis-4-nitrophenyl phosphate, whereas physostigmine had no significant effect (Table 1). The pH measurement of the eluted fractions after running on 170 ml of the Polybuffer displayed a pH gradient which finished at about pH 4.5 (Fig. 1). The highest activity of the two carboxylesterase peaks were eluted at pH 6.1 and

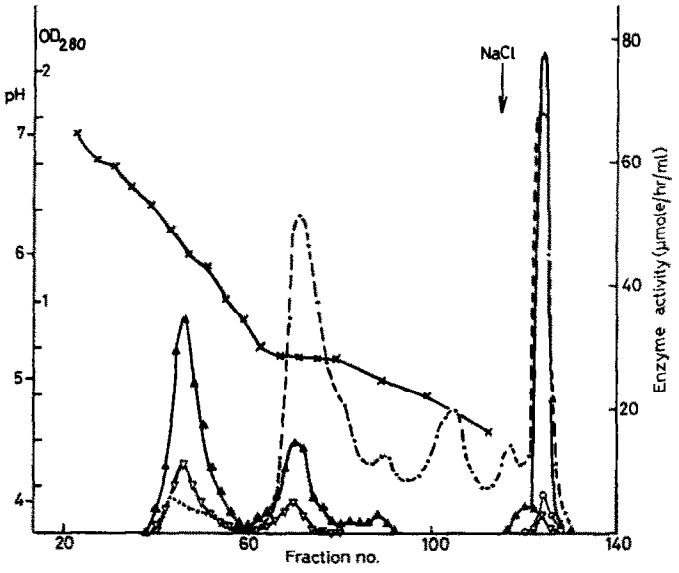

Fig. 1. Chromatofocusing of plasma preparation. The eluted fractions were analyzed for carboxylesterase activity with 4-nitrophenyl butyrate (▲) and methyl butyrate (▽), cholinesterase activity (○), pH (×) and OD_{280} (—·—).

5.2 respectively (Fig. 1), which are quoted as the isoelectric points (Table 1). By a moderate increase in the Polybuffer volume it was possible to extend the pH gradient to pH 4.2, but this was not sufficient to achieve elution of the third carboxylesterase peak with the Polybuffer. Plasma was therefore prepared for electrofocusing to find the isoelectric point of the third carboxylesterase. The fractions eluted during affinity chromatography of plasma displayed activity with 4-nitrophenyl butyrate, but showed unexpected low activity with methyl butyrate as the substrate. After the subsequent electrofocusing only one peak of 4-nitrophenyl butyrate hydrolyzing activity could be detected, as well as one peak of cholinesterase activity (Fig. 2). The pH gradient measured indicated the isoelectric point 4.4 for the cholinesterase and 4.0 for the carboxylesterase (Fig. 2, Table 1).

Separation of liver carboxylesterases

The results obtained by chromatofocusing of the liver preparation are shown in Fig. 3. No cholinesterase activity, two main peaks of 4-nitrophenyl butyrate hydrolyzing activity and three main peaks of methyl butyrate hydrolyzing activity could be detected after elution with the Polybuffer. The second methyl butyrate peak coincided with a reproducible right-sided "shoulder" on the first peak with 4-nitrophenyl butyrate (Fig. 3). No carboxylesterase activity could be detected after elution with the NaCl-solution. The isoelectric point as well as the ratio between activities for the most active fraction of the enzyme peaks are listed in Table 1. The 4-nitrophenyl butyrate hydrolyzing activity of the same three fractions were also measured after preincubation with soman, bis-4-nitrophenyl phosphate and physo-

Table 1. Characteristics of carboxylesterases from guinea-pig plasma and liver—isoelectric point, activity ratio, and effect of selective esterase inhibitors on 4-nitrophenyl butyrate hydrolysis

	pI	Activity ratio 4-nitrophenyl butyrate / Methyl butyrate	Activity (% of control) ± SEM (N = 3–7) Soman 10^{-6} M	BPNP* 10^{-4} M	Physostigmine 10^{-5} M
Plasma	6.1	3.0	1 ± 1	3 ± 1	108 ± 4
	5.2	3.1	6 ± 1	20 ± 2	90 ± 6
	4.0	24.9	10 ± 1	5 ± 2	88 ± 7
Liver	5.7	<13.5	2 ± 1	2 ± 1	61 ± 6
	5.2	4.2	2 ± 1	23 ± 1	96 ± 4
	4.5	26.8	5 ± 2	90 ± 5	98 ± 4

Inhibitors were preincubated with enzyme 30 min 30° at pH 7.8. Control activity (=100%) is without inhibitor.

* BPNP = bis-4-nitrophenyl phosphate.

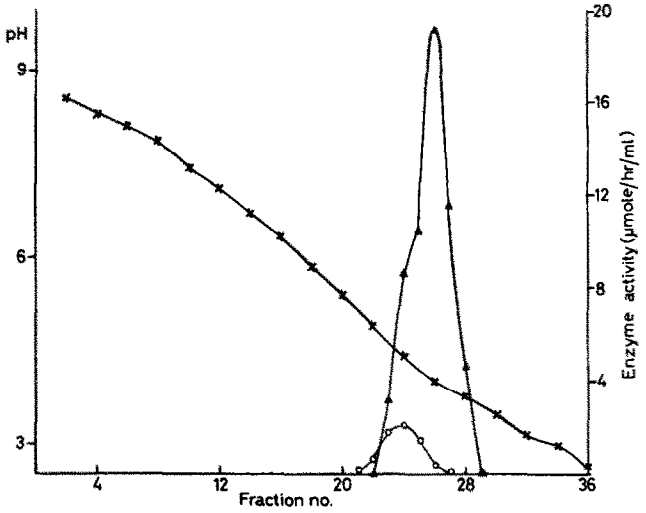

Fig. 2. Isoelectrofocusing of plasma preparation after affinity chromatography. The eluted fractions were analyzed for carboxylesterase activity with 4-nitrophenyl butyrate (▲), cholinesterase activity (○) and pH (×).

stigmine. The compounds showed similar effect as towards the plasma enzymes except for two cases, the pI 5.7 peak activity was partially inhibited by physostigmine and the pI 4.5 peak activity was not significantly affected by bis-4-nitrophenyl phosphate (Table 1).

Reactivation studies

The three carboxylesterase peak activities from both plasma and liver were inhibited at least 90% by soman at the conditions used during reactivation studies. When tested within 2 hr after addition of soman, no significant spontaneous reactivation could be noted. The percentages of oxime-induced reactivation obtained are shown in Table 2. The plasma activities, but not the liver activities, were partially restored when tested 5 min after addition of diacetylmonoxime. During 30 min of incubation no significant increase in the percentages of reactivation could be observed for the plasma activities. The inhibited liver activities were not affected by 30 min of incubation with diacetylmonoxime (Table 2).

DISCUSSION

The intensions of the work was to extend the present knowledge of heterogeneity of carboxylesterase in guinea-pig plasma and liver, and to inves-

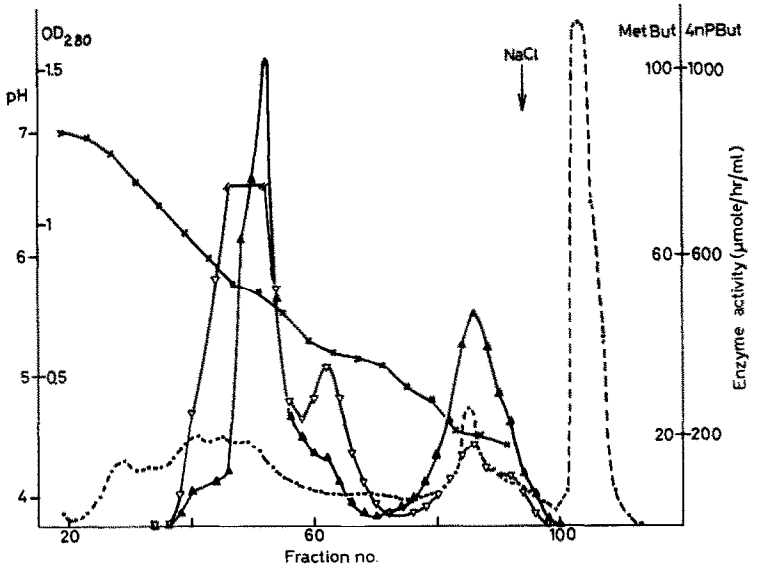

Fig. 3. Chromatofocusing of liver preparation. The eluted fractions were analyzed for carboxylesterase activity with 4-nitrophenyl butyrate (▲) and methyl butyrate (▽), pH (×) and OD_{280} (—·—).

Table 2. Percentages of reactivation by diacetylmonoxime (DAM) of soman inhibited carboxylesterases from guinea-pig plasma and liver

		% reactivation ± SEM (N = 2–6) DAM $9 \cdot 10^{-4}$ M		
	pI	5 min	15 min	30 min
Plasma	6.1	23 ± 3	30 ± 4	29 ± 4
	5.2	62 ± 4	62 ± 5	54 ± 3
	4.0	54 ± 6	53 ± 4	50 ± 3
Liver	5.7	2		3
	5.2	0		2
	4.5	4		3

tigate whether multiple forms of carboxylesterase may differ with respect to reactivatability by diacetylmonoxime after soman inhibition.

Multiple forms of carboxylesterase in both liver and serum of guinea-pig have previously been found to differ with respect to isoelectric point, subunit composition, and substrate specificity (liver) [15, 16]. In accordance, chromatofocusing constituted a convenient method for simple and rapid separation of the carboxylesterase activities in these tissues (Fig. 1, Fig. 3). The isoelectric point of the three main peaks of carboxylesterase activity in plasma (Fig. 1, Fig. 2, Table 1) were near to those reported by Cain *et al.* [16] for two carboxylesterases (pI 6.2, pI 3.9) and an unidentified protein able to bind di-isopropyl phosphorofluoridate but not bis-4-nitrophenyl phosphate (pI 5.0). However, the latter protein of Cain *et al.* was also low in activity towards the substrate phenyl butyrate [16]. This protein might therefore correspond to our small protein peak with low activity towards 4-nitrophenyl butyrate just at pI 5.0 (Fig. 1), which was not further investigated by us. The three main peaks of carboxylesterase activity in plasma (Fig. 1) may all be regarded as carboxylesterases, based on the strong inhibitory power of bis-4-nitrophenyl phosphate (Table 1). This compound is reported to be a specific carboxylesterase inhibitor [19, 20]. This is also in accordance with the strong inhibitory power of soman towards the three enzymes, and with their insensitivity to the cholinesterase inhibitor physostigmine (Table 1). It is interesting in this context that only one of the carboxylesterases, together with the cholinesterase, could be detected after affinity chromatography and subsequent electrofocusing (Fig. 2). The procedure was adapted from Cain *et al.* [16], but we used Affi-Gel Blue 100–200 mesh instead of Blue Sepharose CL-6B for the affinity chromatography.

The isoelectric point of the main peaks of carboxylesterase activity in liver (Fig. 3, Table 1), were for two of them in far agreement with the range (pH 4.6–5.0) reported by Kuhn and Heymann [15] for isoelectric points of one group of carboxylesterases in guinea-pig liver. The three peaks may all be regarded as carboxylesterases, although the pI 4.5 peak was not significantly inhibited by bis-4-nitrophenyl phosphate (Table 1). Since the latter activity was strongly inhibited by soman and not affected by physostigmine (Table 1), the pI 4.5 peak definitely is a B-esterase, but not a cholinesterase. Both with the plasma and the liver enzymes differences in the response to bis-4-nitrophenyl phosphate was observed (Table 1). This is in accordance with the fact that multiple forms of carboxylesterases may be differently affected by this compound [21]. The pI 5.7 carboxylesterase activity in liver was also partially inhibited by physostigmine (Table 1), which is unusual for carboxylesterases. However, effect of physostigmine has previously been reported for pig liver carboxylesterase [22].

Previous results have suggested that methyl butyrate and 4-nitrophenyl butyrate are relevant substrates to use in parallel to reveal heterogeneity of carboxylesterase [14]. In accordance, the results (Fig. 1, Fig. 3, Table 1) showed that the activity ratio towards the two substrates may differ for multiple forms of carboxylesterases within both plasma and liver. Different substrate specificity for carboxylesterases in guinea-pig liver have also been reported by Kuhn and Heymann [15], who compared the substrates methyl butyrate, acetanilide and chloramphenicol.

It is well established that the antidotal effect of diacetylmonoxime for sarin poisoning in several species including guinea pig [23], may be ascribed to its ability to reactivate sarin inhibited carboxylesterase in plasma [24–26]. Previous [8] and present results (Table 2) showed that diacetylmonoxime is also able to reactivate soman-inhibited carboxylesterases in plasma. The results are in accordance with a distinct antidotal effect of diacetylmonoxime for soman poisoning in guinea-pig, which was observed in animals given pyridostigmine prophylaxis together with atropin and toxogonin treatment (Sterri, unpublished). Interestingly, the plasma carboxylesterases were reactivated to a different extent, and none of them was completely reactivated (Table 2). Moreover, none of the liver carboxylesterases was reactivated at the conditions used (Table 2). These results may agree with the findings of Myers [24] that sarin inhibited carboxylesterases within a species are unequal sensitive to reactivation by diacetylmonoxime. The susceptibility of plasma but not liver enzymes to reactivation by diacetylmonoxime (Table 2) might be regarded as unexpected and quite interesting taking into account the usual origin of plasma proteins.

The results (Table 2) may either be due to different ability of diacetylmonoxime to reactivate multiple forms of carboxylesterase, or be a consequence of different rate of aging of soman inhibited carboxylesterases. It is noteworthy, that maximal reactivation of the plasma carboxylesterases were obtained already within 5 min (Table 2). This is much faster than the time (within 30–45 min) for maximal oxime-induced reactivation of soman inhibited acetylcholinesterases [18].

In conclusion, the results show that at least three different carboxylesterases are present in both plasma and liver of guinea pig. The carboxylesterases show both inter- and intra-tissual diversity with respect to reactivatability by diacetylmonoxime after soman inhibition *in vitro*.

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