Biochemical Pharmacology, Vol. 42, No. 7, pp. 1335–1343, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

CARBOXYLESTERASES IN GUINEA PIG

A COMPARISON OF THE DIFFERENT ISOENZYMES WITH REGARD TO INHIBITION BY ORGANOPHOSPHORUS COMPOUNDS *IN VIVO* AND *IN VITRO*

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(*Received* 18 *January* 1991; *accepted* 21 *April* 1991)

Abstract—The different isoenzymes of carboxylesterase (CarbE) from guinea pig liver, lung and plasma were separated by gel filtration and chromatofocusing. The isoenzymes were characterized by inhibition with several different organophosphorus compounds. The bimolecular rate constants showed the same tendency of decreased inhibition for all of the isoenzymes in the order; paraoxon > soman > diisopropylphosphofluoridate (DFP) > bis(*p*-nitrophenyl)phosphate. With two exceptions the inhibition constants for the different isoenzymes differed little. Subcutaneous and intraperitoneal administration of DFP and paraoxon rapidly inhibited the CarbE activity in guinea pig plasma. Much higher doses were necessary to obtain a marked inhibition in lung and liver. About 25% of CarbE activity in lung was resistant to paraoxon and DFP inhibition. Gel filtration of lung homogenate after treatment with the organophosphorus compounds showed that the CarbE activity of the medium molecular mass fractions was inhibited only weakly. This could be due to reduced accessibility to some of the lung CarbE isoenzymes.

Carboxylesterases (CarbEs) (EC 3.1.1.1) are a heterogeneous group of enzymes. The multiple forms are distributed widely in almost all mammalian tissues with the highest concentration of esterase activity localized in the liver. The multiple forms are defined from their isoelectric points and substrate specificity (for reviews see Refs 1–3). Previous work has shown that CarbEs play an important role in the detoxification of different organophosphorus compounds by covalently binding them to their active sites. In rodent plasma especially, CarbEs function as scavengers which remove soman circulating in the blood stream before it reaches the target organs [4, 5].

Injection of 0.5 or 0.75 LD_{50} of soman and sarin highly inhibited the plasma CarbEs but the liver and lung CarbEs were almost unaffected [6, 7]. Also, during inhalation experiments of soman, CarbEs played a small role in detoxification [8]. Experiments on rat liver perfusion with soman showed small effects on liver CarbEs [9]. In this study, we wanted to investigate further why CarbEs of lung and liver, in contrast to those of plasma, are resistant to inhibition by these very active anticholinesterases. We therefore selected compounds such as DFP and paraoxon with a high ratio of CarbE/AChE inhibition to see if we could provoke inhibition of the lung and liver systems. Previous inhibition studies with diisopropylphosphofluoridate(DFP) have shown that the bimolecular rate constant for the reaction with rat lung CarbE is about one hundred times greater than for that with AChE; for paraoxon this ratio is about fifty [10].

Firstly, we separated the different CarbE isoenzymes from guinea pig lung, liver and plasma, and then determined the inhibition constants towards four organophosphorus compounds *in vitro*. Secondly, we injected guinea pigs with DFP and paraoxon to study the inhibition of plasma, lung and liver CarbEs *in vivo*.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained as follows: atropine sulphate from Norsk Medisinal Depot (Oslo, Norway); 4-nitrophenyl butyrate, bis(*p*-nitrophenyl)phosphate and ethopropazine from the Sigma Chemical Co. (St Louis, MO, U.S.A.); Sephadex G-25M, Ultrogel AcA 34, Polybuffer exchanger 94 and Polybuffer 74 from Pharmacia LKB Biotechnology Division (Uppsala, Sweden); acetylcholine iodide from Fluka Chemie AG (Buchs, Switzerland); [1-^{14}C]acetylcholine chloride (55 Ci/mol) from Amersham International (Amersham, U.K.); paraoxon (diethyl-*p*-nitrophenyl-phosphate) from Koch-Light Laboratories (Colnbrook, U.K.). Diisopropylphosphofluoridate and soman (pinacolyl methylphosphonofluoridate) were synthesized in this laboratory and purity was found to be greater than 97% by nuclear magnetic resonance spectroscopy. All other chemicals were of analytical grade quality.

Animals. Male albino guinea pigs, within the weight range of 200 to 400 g, were purchased from the National Institute of Public Health, Oslo. The animals were given a standard laboratory diet and water *ad lib*.

Preparation of guinea pig lung fractions. Guinea

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pig lung (20–30 g) was rinsed with ice-cold 50 mM Tris (pH 7.5) containing 0.1 M NaCl and homogenized in four volumes of the same buffer with a Polytron instrument for 20 sec. The homogenate was filtered through glass wool to remove fat and heated at 55° for 15 min to remove inactive proteins, followed by centrifugation at 9000 *g* for 20 min at 4°. The supernatant was applied to a gel filtration column (Ultrogel AcA 34, 60 × 2.5 cm) equilibrated with 50 mM Tris, pH 8.0. The flow rate was 0.25 mL/min and 4 mL fractions were collected. The fractions were analysed for CarbE. The different molecular weight fractions were further chromatofocused at 4° and separated into isoenzymes.

Preparation of guinea pig liver fractions. Liver from 1–2 animals was rinsed with ice-cold 50 mM Tris (pH 7.5) containing 0.1 M NaCl and homogenized in 4 volumes of the same buffer with a Polytron instrument for 20 sec. The homogenate was filtered through glass wool to remove fat and heated at 55° for 15 min, followed by centrifugation at 9000 *g* for 20 min at 4°. The supernatant was mixed with saponin to a final concentration of 1% (w/w) for 1 hr at 4° and centrifuged at 100,000 *g* for 60 min. The 35–70% ammonium sulphate fraction of the high speed supernatant was desalted by eluting through Sephadex G-25M (Pharmacia PD-10 column) and applied to a chromatofocusing column.

Preparation of guinea pig plasma fractions. The blood from 4–5 animals was transferred to centrifuge tubes and allowed to clot at 37° for 1 hr before being centrifuged at 3000 rpm for 10 min. The supernatant (serum) was removed carefully and diluted 1:1 with 25 mM imidazole–HCl, pH 7.4. The preparation was chromatofocused at 4°.

Chromatofocusing. Lung, liver and plasma preparations were chromatofocused as described in chromatofocusing kit instructions (Pharmacia). A column (0.9 × 28 cm) was packed with PBE 94 and equilibrated with the start buffer, 25 mM imidazole–HCl, pH 7.4. The sample (7 mL) was applied after running 5 mL of the elution buffer, an 8-fold dilution of polybuffer–HCl, pH 3.9. Elution buffer (200 mL) was followed by 2 column volumes of 1 M NaCl solution. The elution rate was 0.40 mL/min and 2.5 mL (or 4 mL) fractions were collected.

Administration of DFP and paraoxon. DFP was diluted in 0.9% NaCl solution and administered either intraperitoneally (0.25–5.0 mg/kg) or subcutaneously (0.25–3.0 mg/kg). Paraoxon was diluted in 0.9% NaCl solution and administered subcutaneously (0.25 and 1.0 mg/kg) together with atropin (5.0 mg/kg i.m.).

Preparation of tissues from guinea pig after DFP and paraoxon administration. Animals were killed by decapitation 1.5 hr after treatment with DFP and paraoxon. The organs (brain, liver and lung) were dissected and 10% (w/v) homogenates of tissue in 20 mM sodium phosphate buffer (pH 7.4) were prepared with a Polytron instrument and with a Potter–Elvehjem homogenizer. Blood with added heparin was centrifuged at 3000 rpm (10 min) for isolation of plasma. The tissue preparations were used undiluted or were diluted in 20 mM sodium phosphate buffer (pH 7.4) before enzyme assay.

Enzyme assay methods. CarbE activity was measured spectrophotometrically with 4-nitrophenyl butyrate as substrate [6].

Total cholinesterase activities were determined by the radiochemical method of Sterri and Fonnum [11]. Acetylcholinesterase(AchE) activity was measured after inhibition of butyrylcholinesterase(BuChE) with ethopropazine [12].

Measurement of inhibition. The isoenzymes of CarbE from guinea pig were incubated with different organophosphates in 0.1 M sodium phosphate buffer (pH 7.8) at 30°. After various time periods (1–50 min) the substrate, 4-nitrophenyl butyrate, was added and the residual activity determined spectrophotometrically [6].

The bimolecular rate constants were calculated according to $k_a = 1/(t \cdot c_1) \cdot \ln(100/x)$ (c_1 = concentration of inhibitor, x = percentage of remaining carboxylesterase activity, t = length of time of preincubation of enzyme and inhibitor before the addition of substrate; Aldridge and Reiner [13]).

RESULTS

CarbEs in guinea pig lung, liver and plasma

CarbEs from the lung preparation were separated by gel filtration into three molecular mass fractions. These three fractions were separated further by chromatofocusing. The high molecular mass fraction consisted of at least two different isoenzymes, pI 4.9 and pI < 4.0, whereas the low molecular mass fraction contained only one isoenzyme, pI 5.9. The medium molecular mass fraction had at least two different isoenzymes with one dominant peak, pI < 4.2 (Fig. 1).

The CarbEs of the liver preparation were separated by chromatofocusing into three different isoenzymes, pI 5.6, pI 5.1 and pI 4.6. A possible fourth isoenzyme was seen as a right-sided "shoulder" at pH 4.4 (Fig. 2).

By chromatofocusing the plasma preparation, two main peaks, pI 6.2 and pI 5.2, of CarbE activity could be detected after elution with Polybuffer. A third isoenzyme, pI < 4.1, was detected after elution with NaCl solution (Fig. 3).

Inhibition by organophosphorus compounds

The rates of inactivation of CarbEs from different guinea pig tissues by organophosphorus compounds are compared in Table 1. The bimolecular rate constant of inhibition was determined from a number of experiments using different concentrations of inhibitor and different incubation times. The inactivation reaction followed first order kinetics since the decrease in log activity was linear with time. One exception was the lung CarbE, pI < 4.2; in the case of two of the inhibitors, bis(*p*-nitrophenyl)phosphate and paraoxon, there was a two phase reaction with two different first order rate constants (Fig. 4, Table 1).

The other isoenzymes of CarbE from guinea pig showed inhibition rate constants in a narrow range (Table 1).

Inhibitory effect of DFP in vivo *in different tissues*

The effects of intraperitoneal administration of

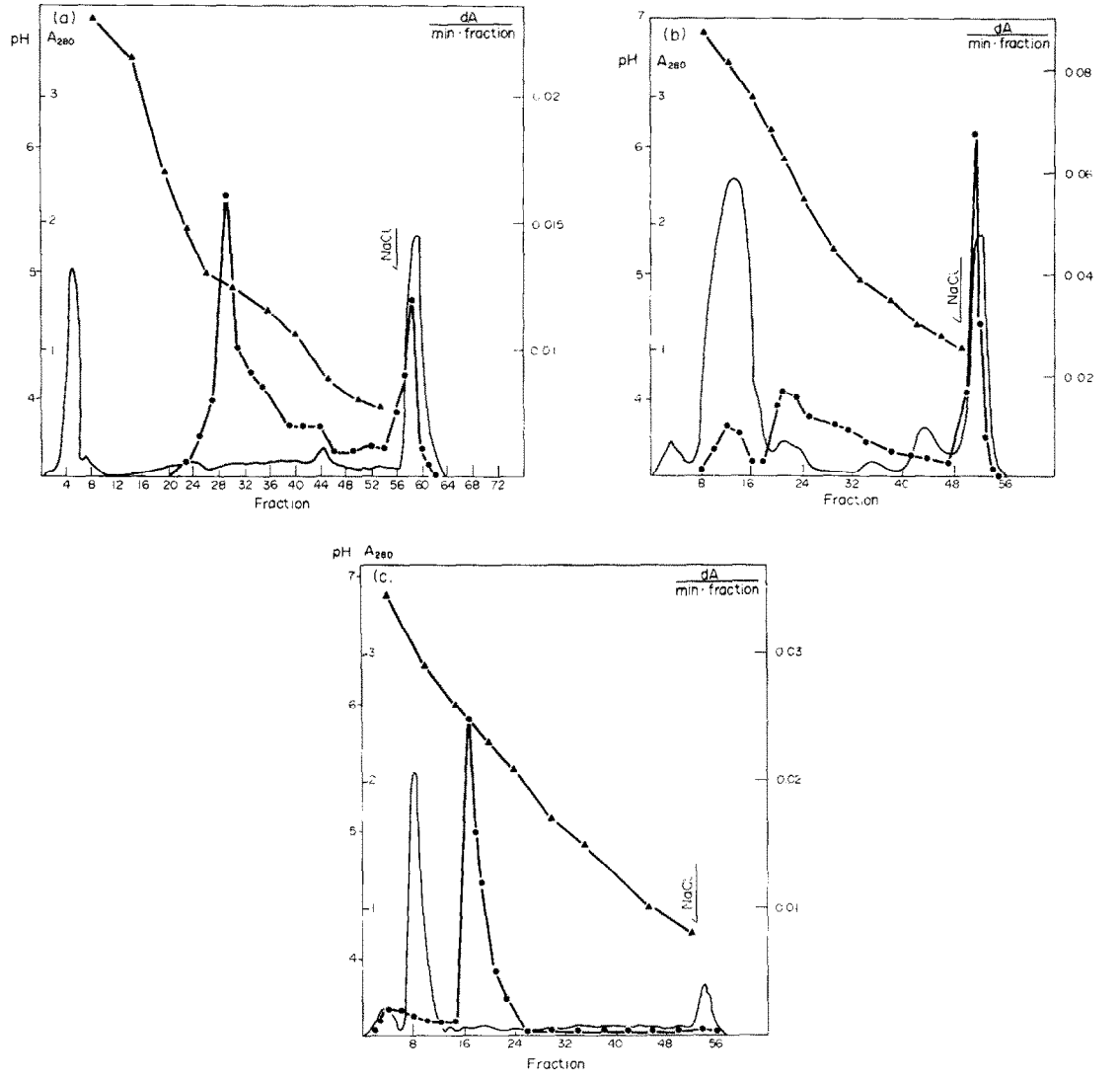

Fig. 1. Chromatofocusing of guinea pig lung after gel filtration. (a) high molecular mass preparation, (b) medium molecular mass preparation, (c) low molecular mass preparation. The eluted fractions were analysed for CarbE activity: dA/min·fraction (•), pH (▲) and A_{280} (−). The column was eluted with 1 M sodium chloride solution after the fraction indicated by an arrow.

0.25–5.0 mg/kg DFP on CarbE and cholinesterase activities in guinea pig lung, liver and plasma are shown in Fig. 5. CarbE activity in plasma was inhibited almost completely at the lowest concentration of inhibitor, whereas lung and liver CarbE activity was inhibited 35% and 50%, respectively, by this dose of DFP. Liver activity decreased with increasing concentration of DFP, whereas 25% of the lung activity was resistant to DFP. A dose of 5.0 mg/kg DFP had to be used to get complete inhibition of the liver CarbE activity. BuChE activity was inhibited to a greater extent than CarbE activity in liver and lung. AChE activity in brain and lung, as expected, was inhibited significantly only at doses greater than 3 mg/kg.

The inhibitory effects of subcutaneous administration of 0.25–3.0 mg/kg DFP on CarbE and cholinesterases activities was also determined (Fig. 6). The inhibition pattern for CarbE activity seen after subcutaneous administration was appreciably different from that seen after intraperitoneal administration of DFP. Only 5% of the activity in the liver was inhibited with the lowest dose of DFP (0.25 mg/kg), whereas 60% of the lung activity was inhibited. When the dose was increased to 3.0 mg/kg ($LD_{50} \approx 3.7$ mg/kg [14]), the liver activity was inhibited almost completely but, again, about 25% of lung CarbE was resistant to inhibition. The cholinesterases were more seriously affected by subcutaneous administration; BuChE activity was inhibited completely, with the exception of liver activity at the low dose. AChE activity in brain and lung was inhibited by almost 80% at a dose of 3.0 mg/kg.

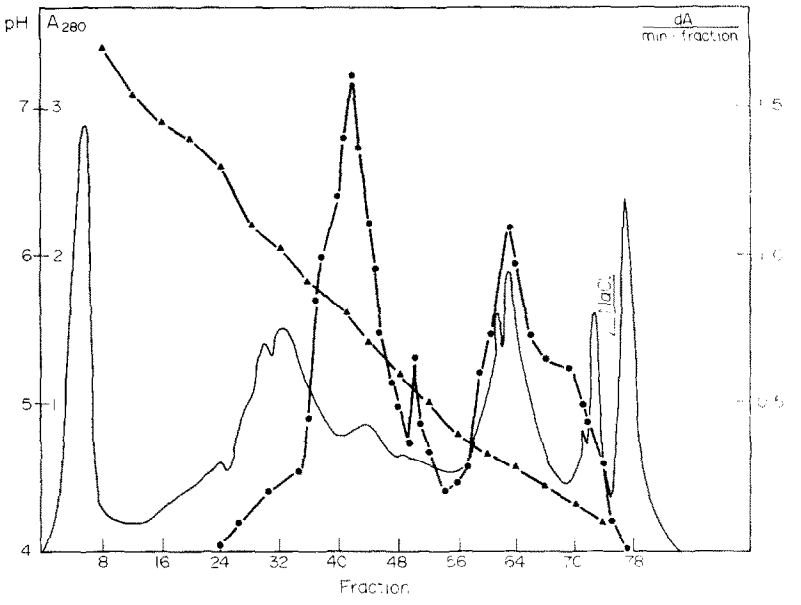

Fig. 2. Chromatofocusing of guinea pig liver preparation. The eluted fractions were analysed for CarbE activity: dA/min·fraction (•), pH (▲) and A_{280} (−). The column was eluted with 1 M sodium chloride solution after the fraction indicated by an arrow.

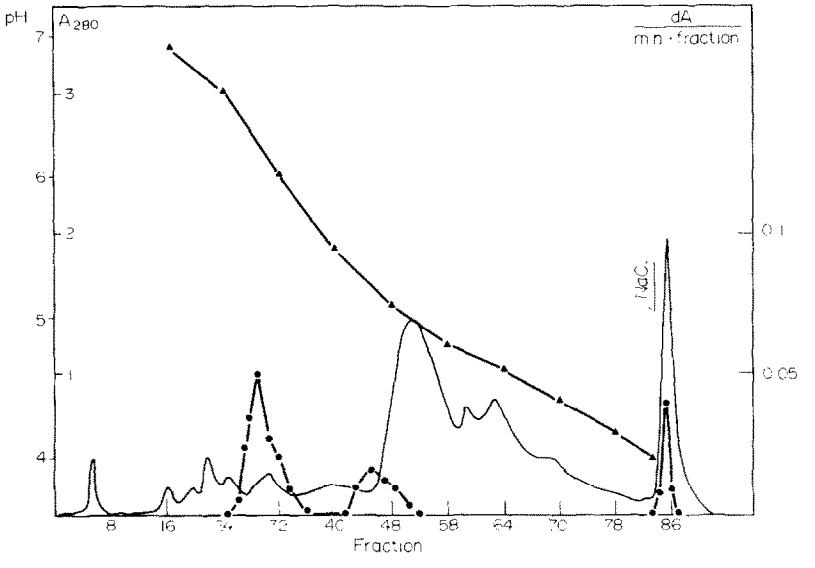

Fig. 3. Chromatofocusing of guinea pig plasma preparation. The eluted fractions were analysed for CarbE activity: dA/min·fraction (•), pH (▲) and A_{280} (−). The column was eluted with 1 M sodium chloride solution after the fraction indicated by an arrow.

Inhibitory effect of paraoxon in vivo *in different tissues*

The specificity of paraoxon as an inhibitor of CarbE, AChE and BuChE in guinea pig is shown in Fig. 7. Doses of 0.25 mg/kg of paraoxon administrated subcutaneously produced an inhibition of nearly 75% of CarbE in lung but this inhibition did not increase when the dose was increased to 1.0 mg/kg ($LD_{50} \approx 0.5$ mg/kg [15]). CarbE activity in the liver was less affected than in the lung, whereas the plasma CarbEs were inhibited rapidly. BuChE activity was much less affected by paraoxon than by DFP.

Comparison of CarbE isoenzyme activities in different tissues after inhibition by DFP and paraoxon in vivo

The CarbE isoenzymes from DFP-treated guinea

Table 1. Bimolecular rate constants for the reaction of carboxylesterases from guinea pig lung, liver and plasma with organophosphorus compounds

Organophosphorus compound	Lung—k_a (M^{-1} min^{-1})			Liver—k_a (M^{-1} min^{-1})			Plasma—k_a (M^{-1} min^{-1})		
	pI 4.9*	pI < 4.2†	pI 5.9‡	pI 5.6	pI 5.1	pI 4.6	pI 6.2	pI 5.2	pI < 4.0
Bis(*p*-nitrophenyl) phosphate	$1.0 \pm 0.1 \times 10^5$	$1.1 \pm 0.2 \times 10^5$§	$9.0 \pm 0.5 \times 10^4$	$1.7 \pm 0.3 \times 10^5$	$1.1 \pm 0.2 \times 10^5$	$2.0 \pm 0.4 \times 10^4$	$6.0 \pm 0.4 \times 10^4$	$8.0 \pm 0.2 \times 10^4$	$1.3 \pm 0.3 \times 10^5$
Soman	$2.2 \pm 0.3 \times 10^7$	$1.9 \pm 0.5 \times 10^7$	$1.3 \pm 0.2 \times 10^7$	$7.9 \pm 0.2 \times 10^6$	$1.1 \pm 0.2 \times 10^7$	$2.0 \pm 0.1 \times 10^7$	$6.9 \pm 0.3 \times 10^6$	$1.1 \pm 0.1 \times 10^7$	$1.0 \pm 0.1 \times 10^7$
Paraoxon	$3.3 \pm 0.3 \times 10^7$	$3.9 \pm 0.3 \times 10^7$‖	$3.9 \pm 0.3 \times 10^7$	$1.9 \pm 0.1 \times 10^7$	$1.7 \pm 0.3 \times 10^7$	$2.7 \pm 0.2 \times 10^7$	$2.1 \pm 0.2 \times 10^7$	$1.3 \pm 0.3 \times 10^7$	$3.6 \pm 0.4 \times 10^7$
Diisopropyl-phosphofluoridate	$2.4 \pm 0.2 \times 10^6$	$2.4 \pm 0.3 \times 10^6$	$6.4 \pm 0.3 \times 10^5$	$1.0 \pm 0.1 \times 10^6$	$1.6 \pm 0.2 \times 10^6$	$2.9 \pm 0.1 \times 10^6$	$5.3 \pm 0.1 \times 10^6$	$4.5 \pm 0.2 \times 10^6$	$4.4 \pm 0.3 \times 10^6$

* High, † medium, ‡ low molecular mass (the most active isoenzymes).
§ Rapid phase. Slow phase, $k_a = 1.0 \times 10^4$ M^{-1} min^{-1}.
‖ Rapid phase. Slow phase, $k_a = 1.0 \times 10^6$ M^{-1} min^{-1}.
Results are means ± SEM (N = 5–8).

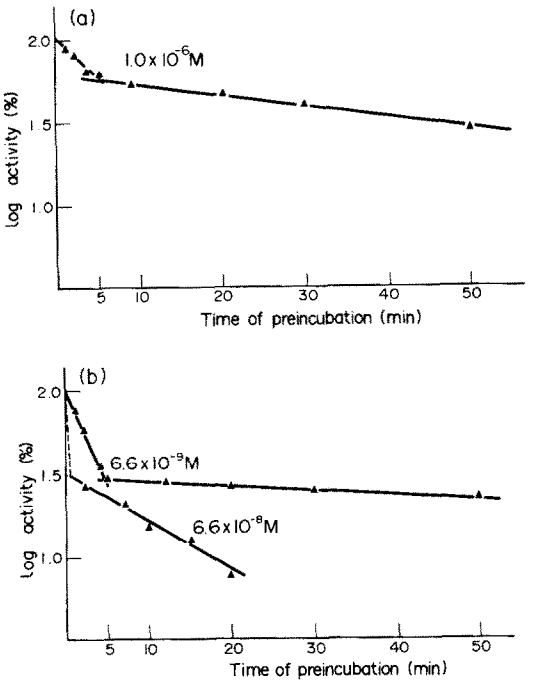

Fig. 4. The rate of inactivation of CarbE, pI < 4.2, from guinea pig lung by (a) bis(*p*-nitrophenyl)phosphate (1.0×10^6 M) and (b) paraoxon (6.6×10^9 M and 6.6×10^8 M). Residual activity was measured after preincubation with the different concentrations of inhibitor.

pig liver and plasma were separated by chromatofocusing. The results showed that after administration of DFP (2–3 mg/kg i.p.), all of the different liver isoenzymes were inhibited to nearly the same extent when compared to those in tissue from untreated animals. The plasma isoenzymes were also equally inhibited, except for CarbE, pI < 4.1, which was inhibited by about 10% less than the other isoenzymes. The lung homogenate was separated by gel filtration into three different molecular mass fractions. In animals treated with 0.25 and 0.5 mg/kg DFP i.p., the high molecular mass fraction was inhibited to a greater extent than the two other fractions. The low molecular mass fraction was more inhibited than the medium molecular mass fraction. In the same way, 0.25 mg/kg paraoxon s.c. inhibited to a great extent the high and low molecular mass fractions, whereas the medium molecular mass fraction was almost completely resistant to inhibition (Fig. 8).

DISCUSSION

The chromatography of lung, liver and plasma homogenates by gel filtration and chromatofocusing illustrates the heterogenity of the CarbEs in the guinea pig. The CarbE activity in guinea pig lung is separated into three molecular mass fractions and each of these has a separate isoenzyme pattern. The results from guinea pig lung are remarkably different from rat lung where the isoenzymes were found

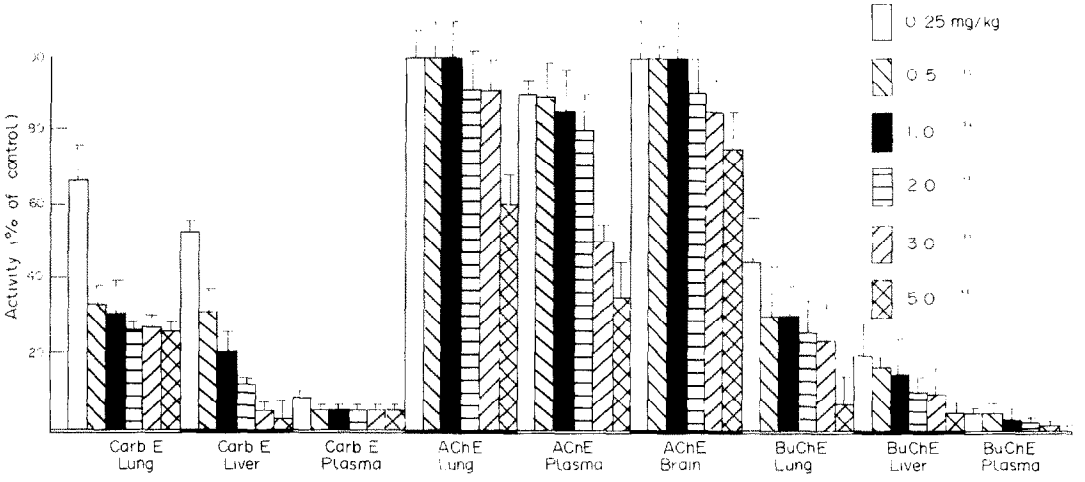

Fig. 5. The effect of intraperitoneal administration of DFP on CarbE, AChE and BuChE activities in guinea pig. (Animals taken for analysis 1.5 hr after DFP injection of 0.25–5.0 mg/kg i.p.) Error bars show SDs of values obtained in assays from four separate animals.

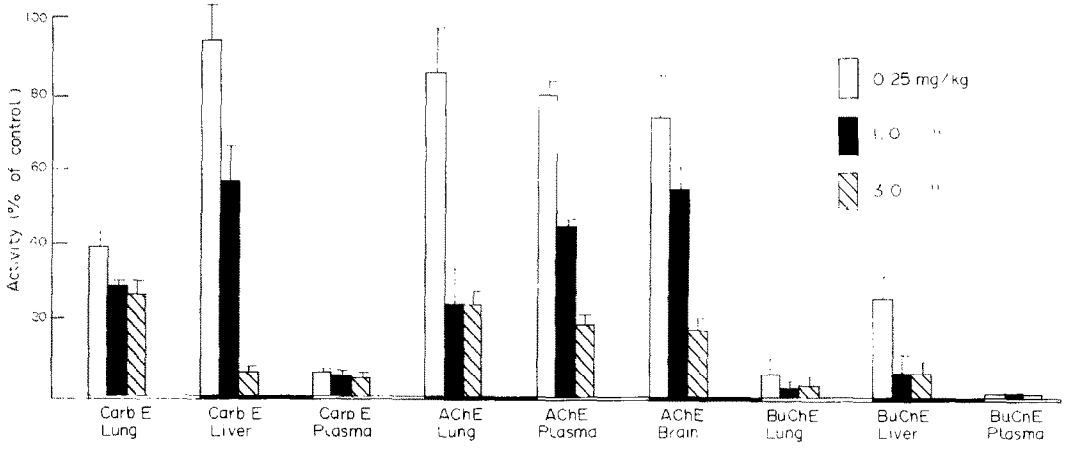

Fig. 6. The effect of subcutaneous administration of DFP treatment on CarbE, AChE and BuChE activities in guinea pig. (Animals taken for analysis 1.5 hr after DFP injection of 0.25–3.0 mg/kg s.c.) Error bars show SDs of values obtained in assays from four separate animals.

within only one molecular mass fraction [10]. The three isoelectric points of the CarbEs in both liver and plasma (Figs. 2 and 3) were close to those reported by Sterri and Fonnum [16].

The various CarbE isoenzymes in liver, lung and plasma appeared to have very similar inhibition rate constants for the four organophosphorus inhibitors examined. There were two exceptions, the lung CarbE, pI < 4.2, and the liver CarbE, pI 4.6. The latter was relatively insensitive to inhibition by bis(*p*-nitrophenyl)phosphate only. The inactivation curves of the lung isoenzyme, pI < 4.2, revealed two different first order reactions for both paraoxon and bis(*p*-nitrophenyl)phosphate (Fig. 4). This indicates two different forms of the isoenzyme with different sensitivities towards the inhibitors. The two inhibitors have the same large leaving group and may behave differently to the other inhibitors. Also, bis(*p*-nitrophenyl)phosphate is the only inhibitor with a negative charge.

The differences between subcutaneous and intraperitoneal administration are illustrated by the inhibition pattern of CarbEs in liver (Figs 5 and 6). These results are in accordance with the observations made by Ramachandran [17], showing that radioactive DFP ($DF^{32}P$) injected into mice or rats by the intraperitoneal route results in a much higher uptake of radioactivity by the liver than when injected by the subcutaneous route. These results can apparently explain the wide gap between the LD_{50}s of DFP injected into mice by the s.c. (3.6 mg/kg) and i.p. (6.7 mg/kg) routes of administration [17]. After intraperitoneal administration, DFP passes first through the liver, whereas after

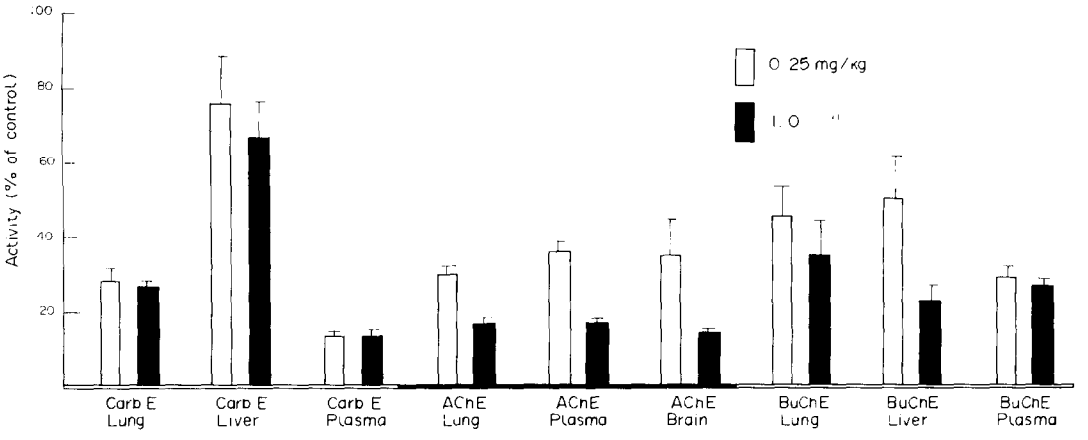

Fig. 7. The effect of subcutaneous administration of paraoxon on CarbE, AChE and BuChE activities in guinea pig. (Animals taken for analysis 1.5 hr after DFP injection of 0.25–1.0 mg/kg s.c.) Error bars show SDs of values obtained in assays from four separate animals.

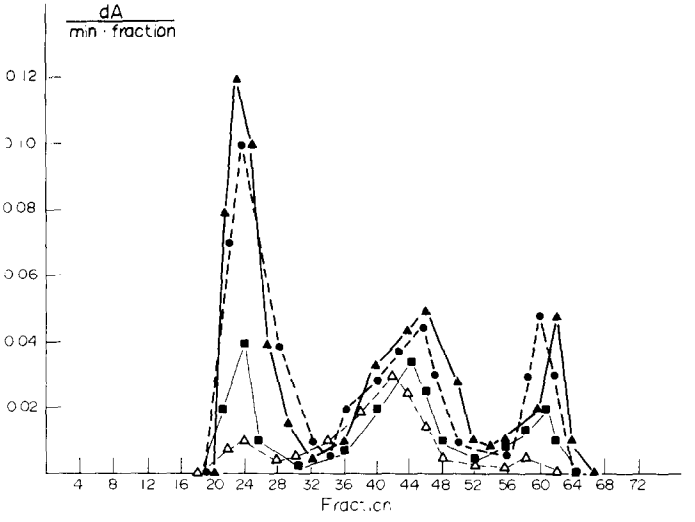

Fig. 8. Representative gel filtration profiles of lung CarbE activity in guinea pig treated *in vivo* with DFP 0.25 mg/kg i.p. (●), 0.5 mg/kg i.p. (■) or with paraoxon s.c. (△). Gel filtration of lung homogenate without any treatment (▲)

subcutaneous administration only about 30% reaches the liver [18].

Enzymes which hydrolyse DFP, like organophosphorus compound hydrolases (OP hydrolases) (EC 3.1.8.1), are present in all tissues of human, rat and guinea pig, the liver containing more of these enzymes than the other tissues [19]. In previous experiments with perfused and hepatectomized rat liver, it was shown that the main part of the agent (soman) was detoxificated through hydrolysis by OP hydrolases before it reached the CarbEs and BuChE [9, 20]. Experiments have shown that different OP hydrolases in rat seems to hydrolyse soman faster than DFP [21, 22]. These OP hydrolases are localized almost exclusively in the soluble fraction of the liver, whereas CarbE activity is found mainly in the microsomal fraction [2]. There is thus the possibility of a poorer accessibility to the CarbEs of soman. The activity of OP hydrolase in guinea pig liver is much lower than that in rat and may not be so important in this species [19].

Unexpectedly, lung CarbE activity was partially resistant to the high doses of DFP. Since the medium molecular mass fraction was inhibited much less than the high and low molecular mass fractions, the explanation for the resistance of a small proportion of lung CarbEs (25%) to organophosphorus inhibitors is likely to be found in this molecular mass fraction. This is the same fraction which showed some insensitivity to paraoxon inhibition *in vitro*. We were not, however, able to detect a similar *in vitro* insensitivity to DFP. This suggests the possibility of different subcellular localizations of the lung CarbE isoenzymes and thereby reduced accessibility, especially for the medium molecular mass fraction isoenzymes. Lung is a highly heterogeneous organ

and consists of at least 40 different cell types [23].

CarbE activity in plasma was inhibited almost completely by the lowest dose of DFP (0.25 mg/kg s.c. or i.p.). This illustrates the importance of plasma CarbE in the detoxification process. Previous experiments *in vitro* have shown that the bimolecular rate constant for the inhibition of porcine serum BuChE by DFP is about 10 times greater than that for rat lung CarbE. For paraoxon, this ratio is reversed [24]. These differences have been partially confirmed by the *in vivo* inhibition experiments (Figs 6 and 7). However, the quantitative importance of BuChE in the irreversible binding of the organophosphorus compound is negligible with respect to detoxification because the concentration of BuChE binding sites is very low (1–2 nmol). In contrast, estimates of the total number of CarbE binding sites have shown it to be over 2000 nmol (based on a 250 g rat) [25].

Hansen *et al.* [26] reported that guinea pig lung rapidly accumulates radioactivity after i.v. administration of tritiated DFP and that the lung acts as a buffer in the initial stages of intoxication. At low doses (0.1 mg/kg) the DFP was bound preferentially to serum and lung. At higher doses (3–6 mg/kg) the DFP accumulated in the liver and kidney. These results are in agreement with our results on the inactivation of CarbE activity seen in plasma, lung and liver. We therefore conclude that the CarbEs in liver and lung represent significant alternative phosphorylation sites for organophosphates at higher doses (>0.5 LD_{50} s.c.).

The efficacy for detoxification is a function of the relative affinities of the organophosphorus compounds for CarbEs and AChE but also of the molar concentrations of the enzymes. It is not possible to assess the contribution of OP hydrolases, but this is less important for guinea pig than for rat. Organophosphorus compounds, like soman and sarin, have higher affinities for AChE than for CarbE [10]. These compounds cause complete inhibition of plasma CarbEs and therefore play an important role as scavengers [5, 7], but the lung and liver CarbEs are not inhibited before the AChE of the brain and muscle is inhibited. In the case of the less potent AChE inhibitors, for example paraoxon and DFP, the lung and liver CarbEs also contribute to the detoxification process.

Acknowledgement—This work was supported by the U.S. Army Medical Research and Development Command (Contract No. DAMD 17-87-G-7004).

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