A CHARACTERIZATION OF CARBOXYLESTERASES IN RAT AND GUINEA PIG - THEIR HETEROGENEITY AND ROLE IN DETOXICATION OF ORGANOPHOSPHORUS COMPOUNDS

A THESIS SUBMITTED TO THE UNIVERSITY OF OSLO FOR THE DOCTOR SCIENTIARUM DEGREE

BY

ROLF GAUSTAD

NDRE/PUBL-93/1001

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The present study deals with the molecular properties of carboxylesterases (CarbEs) and the ability of the various isoenzymes to detoxify organophosphorus compounds. CarbE isoenzymes have been separated from lung, liver, plasma and small intestine of rat and guinea pig mainly by chromatofocusing. One of the isoenzymes in rat lung, pI 5.8, has been purified to near homogeneity. This isoenzymes has a molecular mass of approx 180 kDa with subunits of 60 kDa, and by Edman degradation the first 19 amino acid residues were determined. Monoclonal antibodies (MAbs) were prepared against the purified isoenzyme and used in enzyme-linked immunosorbent assays (ELISA) to distinguish between the CarbE isoenzymes in the different tissues. The molecular and immunological results showed a strong relationship between lung CarbE, pI 5.8, and the rat liver CarbE, pI 6.0.

The MAbs were strongly bound to the high pI forms of the CarbE isoenzymes in plasma and small intestine from rat and guinea pig, but not to the low pI forms. This indicates that at least two immunochemically distinct categories of isoenzymes are persent in both plasma and intestine.

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ABSTRACT (continued)

Furthermore, the properties of the different CarbE isoenzymes with regard to inhibition by selected organophosphorus compounds were studies *in vitro* and *in vivo*. With some exemption the inhibition constants showed small differences between the isoenzymes. Subcutaneous and intraperitonal administration of paraoxon and DFP rapidly inhibited the CarbE activity in guinea pig plasma, much higher doses were necessary to obtain a marked inhibition in lung and liver, and about 25% of CarbE activity in lung was resistant to inhibition. Gel filtration of lung homogenate after treatment with organophosphorus compounds showed that the CarbE activity in one of the molecular mass fractions was weakly inhibited. This might be due to different subcellular localization of the isoenzymes and thereby reduced accessibility to some of the lung CarbE isoenzymes. The experiments showed that the efficacy for detoxication is a function of the relative affinities of the organophosphorus compounds for CarbEs and acetylcholinesterase (AChE). For compounds like paraoxon and DFP, with lower affinities for AChE than for CarbEs, also the lung and liver CarbEs contribute to the detoxication process.

The distribution of CarbEs in separated rat liver cells were investigated by PAGE. All of the liver CarbE isoenzymes could be detected in the parenchymal cells, whereas in both Kupffer cells and endothelial cells only those with pI 6.4/6.2, also termed monoacylglycerol lipase, could be detected.

PREFACE

The work presented in this thesis was carried out at the Norwegian Defence Research Establishment (NDRE), Division for Environmental Toxicology, Kjeller, in the period 1988-1992.

First I want to express my gratitude to Professor Frode Fonnum, Head of Division for Environmental Toxicology, for his support and constructive criticism during this work.

Special thanks are given to Sigrun Sterri, Christian Syversen, Dagfinn Løvhaug, Helge Johnsen, Knut Sletten, Trond Berg and Pål Aas for support and many fruitful discussions and suggestions during the study.

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My thanks are also extended to the entire staff at the NDRE, Division for Environmental Toxicology, for a supporting and friendly attitude.

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Kjeller, April 1993

Rolf Gaustad

Rolf Gaustad

LIST OF ABBREVIATIONS

ACh	-	acetylcholine
AChE	-	acetylcholinesterase
BPNP	-	bis(p-nitrophenyi)-phosphate
BuChE	-	butyrylcholinesterase
CarbE	-	carboxylesterase
DFP	-	diisopropylphosphofluoridate
EC	-	endothelial cells
ELISA	-	enzyme-linked immunosorbent assay
FPLC	-	fast protein liquid chromatography
HAT	-	hypoxanthine, aminopterin and thymidine
HIC	-	hydrophobic interaction chromatography
KC	-	Kupffer cells
MAb	-	monoclonal antibody
PAGE	-	polyacrylamide-gel electrophoresis
PC	-	parenchymal cells
pI	-	isoelectric point
RIA	-	radioimmunoassay
RPC	-	reversed phase chromatography
SDS	-	sodium dodecyl sulphate

CONTENTS

1	**INTRODUCTION**	9
2	**OBJECT OF THE INVESTIGATION**	9
2.1	List of papers	10
3	**GENERAL REVIEW**	10
3.1	A-, B- and C-esterases	10
3.2	Carboxylesterases in different tissues	11
3.2.1	Lung	11
3.2.2	Liver	11
3.2.3	Plasma and monocytes	12
3.2.4	Small intestine	12
4	**DISCUSSION OF METHODS**	13
4.1	Purification procedures	13
4.2	Inhibition studies	13
4.3	Preparation of monoclonal antibodies	15
5	**RESULTS AND DISCUSSION**	15
5.1	Purification and characterization of carboxylesterases from rat lung	15
5.2	The different carboxylesterase isoenzymes with regard to inhibition by organophosphorus compunds *in vitro*	17
5.3	The *in vivo* inhibition of acetylcholinesterase by organophosphorus compounds and the scavenger function of carboxylesterase	17
5.4	Monoclonal antibodies distinguish between carboxylesterase isoenzymes in different tissues of rat and guinea pig	18
5.5	Heterogeneity of carboxylesterases in rat liver cells	20
6	**CONCLUSIONS**	20
	References	21
	Errata	27
Original papers		
Paper I	Purification and characterization of carboxylesterases from rat lung	29
Paper II	Carboxylesterases in guinea pig	37
Paper III	Monoclonal antibodies distinguish between carboxylesterase isoenzymes in different tissues of rat and guinea pig	49
Paper IV	Hetereogeneity of carboxylesterases in rat liver cells	55
	List of distribution	61

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1 INTRODUCTION

Carboxylesterases (CarbEs) (EC 3.1.1.1) are a group of enzymes widely distributed in animals, plants and micro-organisms. They are capable to catalyse the hydrolysis of carboxylic esters, - amides and - thioesters. In addition, CarbEs catalyse the transfer of acyl groups to nucleophilic acceptors, such as aliphatic alcohols or aromatic amines (Junge & Krisch, 1975). They are inhibited stoichiometrically by organophosphates. Because of their wide specificity CarbEs are also known as nonspecific or unspecific carboxylesterases.

Despite detailed biochemical studies, the physiological functions of CarbEs remain mainly unknown. Almost all known substrates are xenobiotics which do not occur normally in the intermediary metabolism. The action of many drugs, e.g., acetylsalicylic acid, cocaine, procaine or atropine is terminated by enzymatic cleavage of their ester bond. Other examples are hydrolysis of anilides of the lidocaine-type of local anaesthetics by liver CarbEs (Junge & Krisch, 1975; Heymann, 1980). These enzymes therefore obviously play a role in detoxication of xenobiotics by their ability to metabolize numerous foreign compounds with ester and amide bonds to a more hydrophilic form with an accelerated excretion. CarbEs are involved in hydrolysis and detoxication of insecticides like pyrethroides and malathion. They are also important in the detoxication of highly toxic organophosphorus compounds through covalent binding of organophosphates to the active sites of the enzymes. It is entirely inefficient by nature to provide detoxication enzymes that are active only towards a single compound, the need is for a broad catalytic activity, as CarbEs, that handle entire groups of compounds and even a variety of reactions. But perhaps the most important function of CarbEs may involve onset and termination of hormone action (Leinweber, 1987). Most steroid hormones exist as fatty acid esters. The estradiol ester acts as an estrogen only after hydrolysis to the free steroid (Hochberg *et al.*, 1990). Esterases that hydrolyze estradiol-17-esters were shown to be present in human breast cancer cells, and they were effectively inhibited by DFP and diethyl-p-nitrophenyl phosphate (Katz et al., 1987).Degradation of juvenile hormones during metamorphosis in insects is an other example of termination of hormone action by esterolytic activity. The juvenile hormone esterase is irreversibly inhibited by paraoxon and support the hypothesis that it is a serine esterase (Abdel-Aal & Hammock, 1986). The necessity of termination of hormone action represents an analogy to bioinactivation of xenobiotic esters.

2 OBJECT OF THE INVESTIGATION

The present study is focussed on the heterogeneity of CarbEs from different tissues of rat and guinea pig, and the aim was to investigate the distribution and properties of the several isoenzymes. Particular interest was paid to the CarbEs in lung, because they may play a role in the detoxication of inhaled organophosphates, and also because lung is one of the tissues with highest activity. Both the charge differences investigated with chromatofocusing and the immunological relationship between CarbEs from different tissues, both in rat and guinea pig, have been used as methods to distinguish between these closely related isoenzymes. A comparative study of the different isoenzymes has been performed with regard to inhibition by organophosphorus compounds both *in vivo* and *in vitro*. Attention was also paid to the distribution of the CarbEs in different cell types in rat liver, where CarbEs are shown to be potential markers

for cell types. A more detailed knowledge of the CarbEs will help to understand the importance of these enzymes in physiological detoxication.

2.1 List of papers

The papers of the present thesis are listed below, and will be referred to in the text by their Roman numerals:

Paper I Gaustad R, Sletten K, Løvhaug D and Fonnum F (1991).
Purification and characterization of carboxylesterases from rat lung.
Biochem J **274**, 693-697.

Paper II Gaustad R, Johnsen H and Fonnum F (1991).
Carboxylesterases in guinea pig - A comparation of the different isoenzymes with regard to inhibition by organophosphorus compounds *in vivo* and *in vitro*.
Biochem Pharmacol **42**, 1335-1343.

Paper III Gaustad R and Løvhaug D (1992). Monoclonal antibodies distinguish between carboxylesterase isoenzymes in different tissues of rat and guinea pig.
Biochem Pharmacol **44**, 171-174.

Paper IV Gaustad R, Berg T and Fonnum F (1992). Heterogeneity of carboxylesterases in rat liver cells.
Biochem Pharmacol **44**, 827-829.

3 GENERAL REVIEW

3.1 A-, B- and C-esterases

The existence of multiple forms, and the wide and frequently overlapping substrate specificities, have posed many problems in the differentiation of esterases. A good classification of esterases, namely A-esterases and B-esterases, are based on their behaviour towards organophosphorus compounds (Aldridge,1953a). A-esterases are not inhibited by organophosphorus compounds but hydrolyse them as substrates. The substituents on the phosphorus can vary widely and the cleavage occurs on the P-O, P-F, P-CN or other bonds; there is some tentative evidence that P-S bonds are also hydrolysed. The name "Organophosphorus compound hydrolase or OP-hydrolase" has now been introduced into the Enzyme Nomenclature with the new number EC 3.1.8.1 for this group. Almost all OP-hydrolases require Ca(II) or Mn(II) for their activity, and EDTA or other chelating agents are therefore strong inhibitors. Hg(II) is also an inhibitor pointing toward the presence of active -SH groups. In contrast, B-esterases, including the CarbEs, are inhibited stoichiometrically by organophosphates through covalent binding to the active site. The mechanism of this reaction is well understood and involves phosphorylation of serine at the catalytic centre. These "serine-esterases" are defined individually (e.g. EC 3.1.1.1 CarbE, EC 3.1.1.7 AChE and EC 3.1.1.8 Butyryl-cholinesterase (BuChE)). AChE and BuChE are distinguished from others due to their preference for cholinesters as substrate. But CarbE and cholinesterases can also be differentiated by using inhibitors: In contrast to cholinesterases, CarbEs are not inhibited by physostigmine at a concentration of 1mM, but are inhibited by bis-(4-nitrophenyl) phosphate (Paper I; Junge & Krisch,1975). A third group of esterases, C-esterases, is neither inhibited nor do they hydrolyze organophosphorus compounds.

3.2 CarbEs in different tissues

3.2.1 Lung

The predominant sites of esterase activity in adult rat lung are the lamellar bodies of the Clara type II cells (Vatter *et al.*, 1968). The alveolar macrophages, which are phagocytic cells found free in the alveoli, have shown intense cytoplasmic staining with α-naphthyl acetate (Xia *et al.*, 1991). There were no previous reports on purification of lung CarbEs from rat or other species. Deimling *et al.*(1983) have by PAGE and isoelectric focusing found at least 8 CarbE isoenzymes in the lung of the house mouse. These esterases account for about 90% of the esterase activity in the lung. In guinea pig lung the CarbE isoenzyme pattern is more heterogeneous compared to the rat lung preparation: The CarbE activity in guinea pig lung was separated into three molecular mass fractions, each with a separate isoenzyme pattern (Paper II). In rat lung the CarbE isoenzymes were found within one molecular mass fraction (Paper I).

3.2.2 Liver

CarbEs have been extensively studied in liver preparations. Almost the entire esterolytic activity of liver may be attributed to B-esterases (Block & Arndt, 1978; Mentlein*et al.*,1980). By labelling liver slices with [3H]-diisopropylfluorophosphate it has been demonstrated that the bulk of organophosphate sensitive esterase sites are localized in the hepatocytes (Milz & Budd,1978). Results from our laboratory, however, have shown that both Kupffer cells and endothelial cells also contain CarbE activity (Paper IV). The CarbE activity in liver is mainly localized in the microsomal fraction, but occasionally, the bulk of CarbE activity is found in the soluble fraction. This is probably a result of autolyse or of the special separation technique. However, cytosolic CarbE, with different properties from the microsomal CarbE isoenzymes, has been identified. Three reports have been published about specific cytosolic liver serine esterases in man, rat and guinea pig capable of hydrolysing acetylsalicylic acid with different properties from the microsomal enzymes (Williams *et al.*,1989; Kim *et al.*,1990; White & Hope, 1984), and Mentlein *et al.*(1987) have identified some cytosolic activity with differing electrophoretic properties. It has been suggested by some authors that the cytosolic esterases are merely a contamination from the microsomal enzymes. Lately Robbi and Beaufay have in two articles shown that the COOH-end targets the CarbE to the lumen of the endoplasmatic reticulum (ER) (Robbi & Beafay,1991; 1992). The C-terminal sequence His-Ile-Glu-Leu of rat liver CarbE, pI 6.0, contains the C-terminus Glu-Leu, which appears to be the most stringent requirement for retention of the protein in the ER. If the C-terminus is mutated to Glu-His-Thr, the rat liver isoenzyme is secreted. Medda and Proia (1992) have shown that one member of the rat liver CarbE family contains this C-terminal retention signal and probably represents a secreted form. The development of the capacity of CarbEs to be targeted to both intracellular and extracellular locations may also have served to increase the effectiveness of the detoxication system in order to handle the myriad of foreign compounds.

The liver of mammals contains a variety of CarbEs with different substrate specificity. Six microsomal CarbEs/amidases have been isolated from rat liver microsomal fractions (Mentlein *et al.*,1980; Robbi & Beufay,1983). These enzymes have been classified according to their isoelectric points (CarbEs pI 6.4, 6.2, 6.0, 5.6, 5.2, 5.0). The CarbEs pI 6.2 and 6.4 seem to have almost identical catalytic properties and they hydrolyse long-chain thioesters with maximum activity for palmitoyl-CoA (Berge,1979; Mentlein*et al.*,1984a; Robbi & Beafay,1988). CarbE, pI 6.0, represents about 2 % of the total microsomal protein in rat liver (Heymann*et al.*,1979). This isoenzyme has the highest activity towards short aliphatic esters such as methylbutyrate, and also towards amides such as butanilicaine (Mentlein*et al.*,1980). CarbE, pI 5.6, shows three bands on polyacrylamide gelelectrophoresis which corresponds to heterogeneity at the N-terminal end. The isoenzyme has been described as an acylcarnitine-cleaving enzyme (Mentlein *et al.*,1985a), but the isoenzyme has high activity towards acetanilide (Mentlein & Heymann,

1984). The esterases termed pI 5.2 and pI 5.0 represent genetic variants, but their catalytic properties hardly differ (Mentlein *et al.*,1987). The spesificity for these isoenzymes is a high hydrolase activity towards medium-chain acylcarnitines, but also a remarkable activity towards diglycerides. These acylcarnitine hydrolases could be involved in the transport of carnitine out of the liver (Mentlein *et al.*,1985). The CarbE isoenzymes, pI 5.6 and pI 5.2/5.0, have shown hydrolyzing activity towards 12-*O*-tetradecanoylphorbol-13-acetate and 1-oleoyl-2-acetyl-rac-glycerol, both activators of protein kinase C which has been suggested to be involved in carcinogenesis and cell proliferation. The isoenzymes might prevent the accumulation of these activators in rat liver by converting them to inactive forms and thereby indirectly block the activation of protein kinase C (Mentlein, 1986; Maki *et al.*, 1991). All of the liver CarbE isoenzymes appear with similar subunit mass of approximately 60 kD, and they behave as monomers, except for CarbE, pI 6.0, which appears with a trimeric structure (molecular mass of 180 kD) (Mentlein *et al.*,1980). The classification of the rat liver CarbEs according to their isoelectric points is not always satisfactory because the same isoenzyme in another strain of rat may have a different pI. Thus, it is preferable to name these esterases according to one of their activities, and if possible, on natural intracellular substrates. Parallel to biochemical discrimination between rat liver CarbEs, a system for genetic classification of rat esterases has been developed (Simon *et al.*,1985; Mentlein *et al.*,1987). The nomenclature, recommended by Van Zutphen (1983), is based on genetic variation of esterase isoenzymes and may be detected electrophoretically (e.g.,ES-3 represents pI 5.6).

3.2.3 Plasma and monocytes

Several papers in our laboratory have demonstrated that CarbE in rodent plasma is able to function as a scavenger of nerve agents (Sterri, 1981; 1989; Sterri & Fonnum, 1984; 1989; Sterri *et al.*,1980; 1981; 1985a; Fonnum & Sterri, 1981; Fonnum *et al.*,1985). The heterogeneity of CarbEs has also been shown in the plasma: In rat and guinea pig plasma, respectively, two and three different CarbEs have been reported (Choudhury, 1974; Hashinotsume *et al.*,1978; Cain *et al.*,1983; Sterri & Fonnum, 1987; 1989; Paper III). Lately Van Lith *et al.* (1989, 1991, 1992a) have described three different rat plasma CarbEs (ES-1, ES-2, ES-14) with molecular masses of 55.5, 61.1 and 65.3 kD respectively. The isoenzyme ES-1 represents very little of the total plasma esterase activity, but the ES-1 activity was found to be increased with increasing fat intakes. Human monocyte CarbE has been purified to homogeneity by Saboon and Newcombe (1990). The purified enzyme showed, as many other CarbEs from different tissues and species, a trimeric structure and a subunit mass of 60 kD. Inhibition of the human monocyte CarbE by organophosphorus compounds has been used as a biomarker of organophosphorus exposure (Emmett *et al.*,1985). The absence of monocyte CarbE in some humans has led to the speculation that these humans are at increased risk for cancer, because of reduced detoxication of toxic organic esters (Markey *et al.*,1987).

3.2.4 Small intestine

The small intestine represent a major depot of CarbE, and the relative CarbE activity in rat duodenum is about as high as in liver when the substrate 4-nitrophenyl butyrate was used (Fonnum *et al.*,1985). The heterogeneity of the CarbEs in the small intestine have been studied in rat and guinea pig (Sterri, 1989; Paper III). Experiments with CarbEs by light microscopy of small intestine from different mammals have shown an unexpectedly uniform pattern (Böcking *et al.*,1976). Because of similarities in oxime-induced reactivatability following organophosphorus inhibition, in isoelectric points and in immunological relationship, it has been proposed that the intestinal CarbE may be a possible source of the enzyme in plasma (Sterri,1989; Böcking & Deimling,1982; Paper III).

4 DISCUSSION OF METHODS

4.1 Purification procedures

The CarbEs are very stable enzymes, and this property was utilized in the purification procedure by heating the homogenate to 55°C for 15 min to remove heat labile proteins (Paper I). Whereas CarbEs had to be solubilized from rat liver microsomal membranes by treatment with a detergent like saponin (Mentlein *et al.*,1980), the CarbE activity of rat lung was mainly located in the cytosolic fraction, and the yield of solubilized enzyme did not increase after use of detergent (Paper I). The solubilized proteins were precipitated with ammonium sulphate and separated by gel filtration. The most difficult part in the purification is to separate the mixture of closely related isoenzymes. Procedures like isoelectric focusing and chromatofocusing have proved to be highly successful methods for separating proteins according to their isoelectric points. Chromatofocusing was used in the separation of the CarbE isoenzymes (Paper I, II & III). This method is a simple technique to set up and samples containing several hundred milligrams of protein can be processed in one step. The further purification may involve steps as anion- and cation-exchange chromatography, but also hydrophobic interaction chromatography. Ion exchange chromatography with Mono Q and Mono S was excellent in the purification of the lung CarbE, pI 5.8, to near homogeneity. MonoBeads (the name is derived from the unique monodisperse nature of the matrix) ion exchangers are based on a 10 μm beaded hydrophilic polyether resin which have been substituted with quaternary amino groups to yield the strong anion exchanger, Mono Q, or with methyl sulphonate groups to yield the strong cation exchanger, Mono S. High efficiency, coupled with the excellent selectivity of the Q and S substituents results in the high resolution separations characteristic of FPLC technology. Hydrophobic interaction chromatography (HIC) was used in the last step of the purification. Use of HIC can be advantageous following ammonium sulphate precipitation, since samples are normally applied in high salt concentrations. But in our situation the protein concentration after ammonium sulphate precipitation was too high to be loaded on the small HIC column using the FPLC system. Unlike reversed phase chromatography (RPC), in which hydrophobicity is also the mechanism of separation, HIC does not require the use of organic solvents which CarbEs is specially sensitive to.

Various patterns of isoenzymes of CarbE may be obtained depended on the substrate used for detection. In the experiments performed in this thesis the substrate 4-nitrophenyl butyrate has mostly been used. The activity towards this substrate has shown no sex differences, and the substrate may be ideal in monitoring the CarbEs which are most important for the detoxication of organophosphorus anticholinesterases like soman (Sterri *et al.*,1985b).

4.2 Inhibition studies

The CarbE isoenzymes and inhibitors were preincubated under defined conditions of pH, ionic composition and temperature. After various times of preincubation, substrate was added to stop the reaction and the residual activity determined spectrophotometrically with 4-nitrophenyl butyrate as substrate. The results were plotted as time of inhibition versus log of residual activity. From these graphs it was possible to deduce that the rates of inhibition followed first order kinetics. Furthermore the rate constants were linearly related to the concentration of inhibitors. The bimolecular rate constant was then derived by plotting the inhibitor concentration against the first order rate constant (see Figure 4.1). The product of the time for 50% inhibition and the concentration of the organophosphorus compound has shown to be a useful constant. The relationship between the concentration of inhibitor c_i and the time of inhibition t can be given by:

$$k_a = [\ \ln(100/x)\ ] / (t \cdot c_i)$$

where k_a is the bimolecular rate constant and x is the percentage of esterase activity remaining (Aldridge & Reiner,1972). This relationship was used in the calculations of the bimolecular rate constants in Paper I & II, but for some of the inhibitors the bimolecular rate constants were also determined from the plotting procedure (Figure 2.1) as a control.

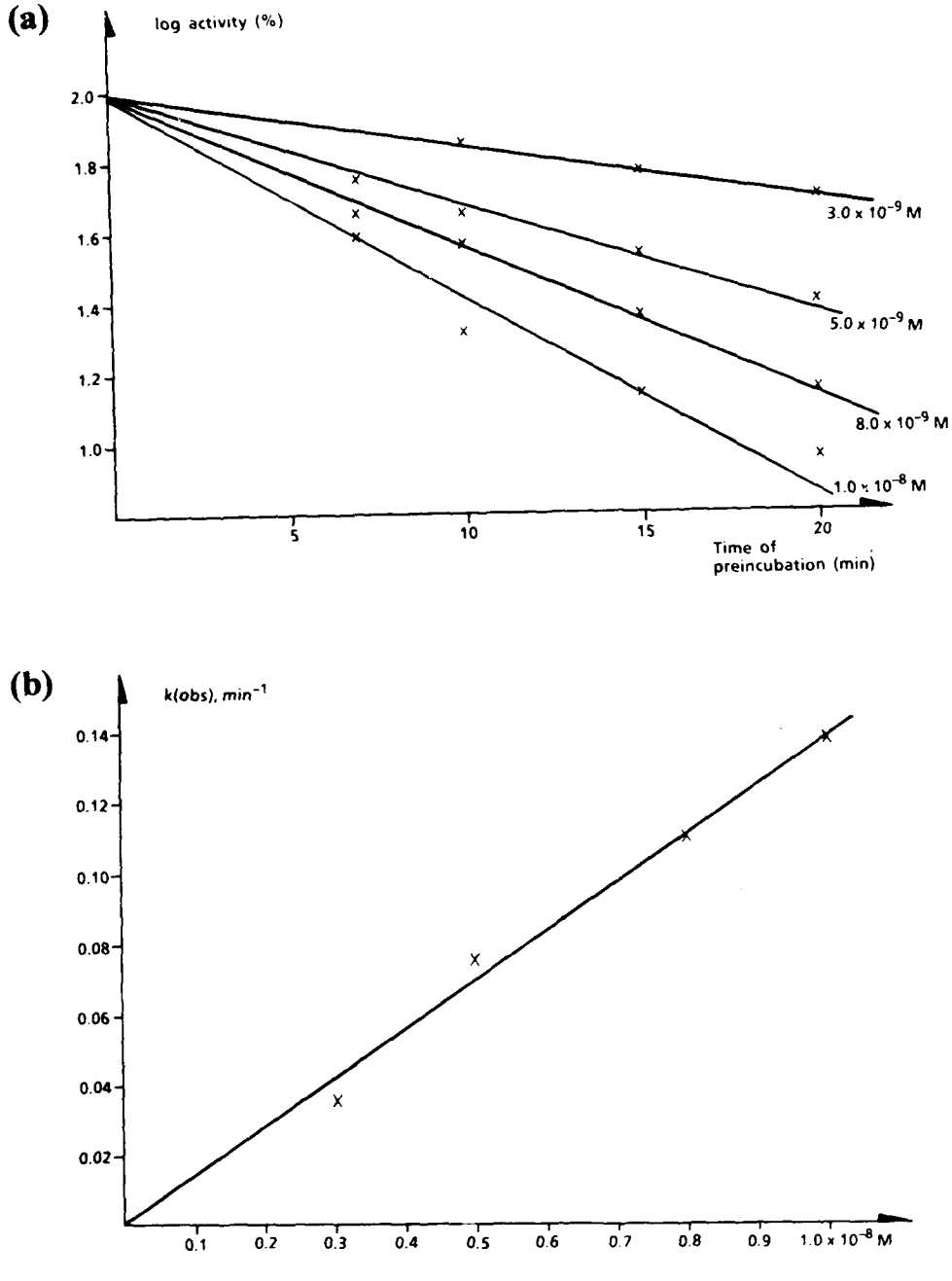

Figure 4.1 The rate of inactivation of rat lung CarbE (pI 5.8) by paraoxon
a) Residual activity after preincubation with different concentration of inhibitor
b) The relation between the first order rate constant, k(obs), and the inhibitor concentration

4.3 Preparation of monoclonal antibodies

The first reported derivation of cell lines capable of permanent production of specific antibody directed against a predefined immunogen was by Köhler and Milstein (1975). The method was based on fusion of myeloma cells and spleen cells from suitable immunized animals by addition of polyethylene glycol which promotes membrane fusion. Spleen cells die in a short time under ordinary tissue culture conditions. Myeloma cells have been adapted to grow permanently in culture, and mutants were isolated that lacked the enzymes hypoxanthine guanine ribosyltransferase or thymidine kinase. The mutants cannot grow in medium containing aminopterin (HAT medium). But hybrids between such cells and spleen cells can be selected from the parental components as the only cells that actively multiply in HAT medium. These fused cells survive as they have the immortality of the myeloma cells and the metabolic bypass of the spleen cells. To ensure the monoclonality of the antibodies and to get rid of non-producer cells, cloning by limiting dilution are used. The cells were diluted to less than one cell per well in microculture plates and the positive wells were selected (see Paper III). Enzyme linked immunosorbent assay (ELISA) was used to analyse for antibody production, and was also used in the experiments analysing for immunological relationship between CarbEs from different tissues and pecies. The main principle of the ELISA technique is that in the final step of the assay, the anti-immunoglobin is enzyme labelled instead of radioiodinated as in radioimmunoassay (RIA). The degree of antibody binding is evaluated through the colour development that follows after addition of substrate to the system. Although ELISA is somewhat less sensitive than RIA, the handling of samples, colour development and its measurement are faster, especially with the automatic scanners. In the procedure of ELISA the choice of immuno plates and blocking solution were of great importance for the results. Nunc Immuno plates were shown to be the best system for coating antigen to polystyrene. The plates were blocked with excess of 10% fetal bovine serum to prevent any subsequent non-specific binding of proteins.

5 RESULTS AND DISCUSSION

5.1 Purification and characterization of carboxylesterases from rat lung (Paper I)

CarbE from rat lung was separated by chromatofocusing into three different isoenzymes. The most active isoenzyme, pI 5.8, was purified to near homogeneity, corresponding to a single band on SDS/PAGE with a monomer mass of 60 kDa. The molecular mass was determined to be 180 kDa and we therefore assume a trimeric structure. These results are similar to those obtained with rat liver CarbE, pI 6.0 (Mentlein *et al.*,1984).

Comparison of the N-terminal amino acid sequence of a 60 kDa glycoprotein esterase from rabbit liver (Korza & Ozols, 1988) and the N-terminal region of rat lung CarbE, showed differences in positions 1, 4 and 19. Later, Robbi *et al.* in 1990 identified the nucleotide sequence of cDNA coding for rat liver pI 6.0 esterase (ES-10). A comparison of the 19 residues of the N-terminal sequence from rat lung with this sequence, only showed a difference in position 9 (Asn was exchanged with Asp). Again we observed a high degree of similarity between these two CarbE isoenzymes. Sequence analysis of the N-terminal sequence of the rat lung CarbE, pI 5.8, showed partial analogy with the N-terminal sequence of *Torpedo* AChE (see Table 5.1).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
(1)	Tyr	Pro	Ser	Ser	Pro	Pro	Val	Val	Asp	Thr	Val	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr
(2)	Tyr	Pro	Ser	Ser	Pro	Pro	Val	Val	Asn	Thr	Val	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr
(3)	His	Pro	Ser	Ala	Pro	Pro	Val	Val	Asp	Thr	Val	Lys	Gly	Lys	Val	Leu	Gly	Lys	Phe
(4)	Asp	Asx	His	Ser	Glu	Leu	Leu	Val	Asn	Thr	Lys	Ser	Gly	Lys	Val	Met	Gly	Thr	Arg
(5)			Glu	Asp	Asp	Ile	Ile	Ile	Ala	Thr	Lys	Asn	Gly	Lys	Val	Arg	Gly	Met	Asn

Table 5.1 *Sequence comparison of the N-terminal regions of (1) rat lung CarbE (pI 5.8) (Paper I), (2) rat liver CarbE (pI 6.0) (Robbi et al.,1990), (3) 60 kDa rabbit liver microsomal esterase (Korza & Ozols, 1988), (4) Torpedo AChE (MacPhee-Quigley et al.,1985) and (5) human BuChE (Lockridge et al., 1987). Residues underlined represent differences in the sequence of (2), (3), (4), and (5) compared to (1)*

The results with the substrate-specificity of the CarbE, pI 5.8, showed a high activity towards esters with an aromatic alcohol group. The aromatic amides, as typical for CarbEs, were hydrolysed at a much lower rate. But the highly purified CarbE from rat lung showed higher rate constant (V/Km) for butanilicaine compared to two reports (Arndt & Krisch, 1973; Mentlein & Heymann, 1984) using rat liver CarbE, pI 6.0 (see Table 5.2). But for pig liver CarbE (corresponding to rat liver, pI 6.0) the rate constant was greater than for the lung CarbE.

CarbE	4-Nitrophenyl acetate		4-Nitrophenyl butyrate		Metyl butyrate		Butanilicaine	
	Km	V	Km	V	Km	V	Km	V
Rat lung (1) pI 5.8	0.06	107	0.02	64			0.43	15
Rat liver (2)	0.13	66	0.25	19	0.6	520	0.25	5.0
pI 6.0 (3)					0.21	454	0.97	24
pI 5.6 (3)	0.16	30			0.45	36	3.4	5.1
Pig liver (2)	0.043	110			0.45	700	0.60	57

Km (mM) V (μmole/min/mg)

Table 5.2 *Kinetics of the hydrolysis of esters and amides by different CarbE isoenzymes*
(1) Data from Paper I
(2) " " Heymann, 1980
(3) " " Mentlein et al., 1984a

5.2 The different CarbE isoenzymes with regard to inhibition by organophosphorus compounds in vitro (Paper I & II)

The rate of inactivation of the different CarbE isoenzymes from rat lung and guinea pig liver, lung and plasma by organophosphorus compounds showed small differences (Paper I & II). The bimolecular rate constants decreased in the following order; paraoxon > soman > diisopropylphosphoflouridate (DFP) > bis(p-nitrophenyl)-phosphate (BPNP). For the inhibition of AChE by the same compounds this order differ (see Paper I, Table 5.3). The rate constants for both esterases are increased when the alkoxy group is lengthened. Inhibition of AChE by organophosphates with two bulky alkoxy groups, as paraoxon and DFP, showed low rate of inactivation. BPNP is only a very weak inhibitor of AChE. As mention in paper I, the negative charge on this inhibitor probably prevents binding to the esteratic locus of the AChE active site because of the nearby anionic locus. The differences in rate of inactivation have to imply main diversity between CarbE and AChE in their active sites. As BPNP is a good inhibitor of CarbE, one can exclude any anionic locus in the CarbE active site. Later Sussman *et al.* (1991) have determined the three-dimensional structure of AChE from *Torpedo californica*. They have shown that the active site lies near the bottom of a deep and narrow gorge that reaches half way into the protein. By modelling of ACh into the enzyme, the quaternary ammonium ion is bound not to a negatively charged anionic locus, but rather to some of the fourteen aromatic residues that line the gorge.

5.3 The in vivo inhibition of AChE by organophosphorus compounds, and the scavenger function of CarbE (Paper II)

The primary target for organophosphorus compounds, such as insecticides and nerve agents, is the inhibition of acetylcholinesterase (AChE). The symptoms accompanying poisoning can be explained by an increase in the level of ACh, in brain up to about 100% (Fonnum & Guttormsen,1969), and thereby a large excess of ACh at the cholinergic receptors and an overstimulation of the cholinergic system.

The *in vivo* inhibition of AChE by organophosphorus compounds will vary dependent on physiological processes like:

1) The route of administration, whether subcutaneous, intraperitoneal or by inhalation, will influence the distribution of organophosphates to the target organs. After intraperitoneal administration, the organophosphate passes first through the liver, but after subcutaneous administration only about 30% reaches the liver (Gaines *et al.*,1966). The relative high activity of detoxication enzymes like CarbE and OP hydrolases in liver (Maxwell *et al.*,1987) results in a significant difference between the LD_{50}s obtained by the two different routes of administration (Ramachandran,1966; Fonnum & Sterri,1981). After inhalation of organophosphates like soman the CarbE in lung is more inhibited compared to experiments with injection of organophosphates (Walday *et al.*,1991; Sterri *et al.*,1985b).

2) The delivery of organophosphates to tissues by blood flow. The total tissue blood flow in lung and hepatic arterial blood flow in liver are of very different values; 30 ml/min/g for lung and 0.78 ml/min/g for liver (Bischoff *et al.*,1971).

3) A catalytic detoxication of organophosphates by organophosphorus (OP) compound hydrolases. OP-hydrolases are present in all organs in mammals with highest activities in liver and blood (Aldridge, 1953b; Chemnitius *et al.*,1983). The influence of the hydrolases in detoxication is dependent on the activity and the distribution of special types of OP-hydrolases in the different tissues (like somanase, DFPase or paraoxonase).

4) A noncatalytic detoxication by CarbE. As mentioned before a series of articles in our laboratory have shown that CarbEs in plasma of rodents are able to function as scavengers which remove organophosphates circulating in the blood stream before they reaches the target organs. Human plasma contains no CarbE (Aldridge, 1953a; Christen *et al.*, 1969). The difference in LD_{50} of soman between different species correlates with the difference in plasma concentration of CarbE (Sterri & Fonnum, 1989).

Different *in vivo* experiments have confirmed the contribution of CarbE in the detoxication process:

- Intravenous administration of CarbE lowered the toxicity of soman in young rats (Fonnum *et al.*,1985).
- Pretreatment of mice with phenobarbital increased the LD_{50} value significantly, as a result of an elevation of both serum and liver CarbE activity (Clement, 1984).
- Injection of TOCP (tri-ortho-cresyl phosphate) and DBDP (2-(2-methylphenoxy)-4H-1,3,2-benzodioxaphosphorin-2-oxide), irreversible inhibitors of CarbE (Mendel & Myers, 1953; Polak and Cohen, 1969), increased dramatically the acute toxicity of soman (Sterri *et al.*, 1981; Clement, 1984).

The large activity of CarbE in liver and lung was also shown to play an important role as scavenger for organophosphates with higher affinities for CarbE than for AChE (Paper II).

BuChE will have a negligible role since the number of esteratic sites available for detoxication by irreversible binding are very low (Maxwell *et al.*,1987).

5.4 Monoclonal antibodies distinguish between CarbE isoenzymes in different tissues of rat and guinea pig (Paper III)

The CarbE isoenzymes from the tissues of rat and guinea pig were separated by chromatofocusing. The isoenzyme pattern illustrates the heterogeneity of the CarbEs in these tissues (lung, liver, plasma and small intestine). A comparison between the pI values for the CarbEs in liver and plasma for both rat and guinea pig shows large variations, whereas the same comparison for small intestine and plasma CarbEs display more similar values, especially for low pI values (see Table 5.3).

Two monoclonal antibodies (MAbs) raised against rat lung CarbE, pI 5.8, were used as probes to distinguish between the closely related isoenzymes. The immunological results for the reaction between the two MAbs and the CarbEs from small intestine correspond well to the same reaction with the plasma CarbEs for both rat and guinea pig (Table 5.4). In both plasma and small intestine of guinea pig three different CarbE isoenzymes have been identified. The pI and the binding to the MAbs for these CarbEs corresponded closely. In rat plasma we have identified two CarbE isoenzymes which corresponded in both pI and MAb binding with isoenzymes from rat small intestine.

Rat CarbEs (pI):												
Lung			Liver				Plasma		Small intestine		
5.8	5.5	5.3	6.4/6.2	6.0	5.6	5.2/5.0	4.4	<4.0	5.4	4.8	<4.2

Guinea pig CarbEs (pI):												
Lung					Liver			Plasma			Small intestine	
4.9	<4.0	5.8	<4.2	5.9	5.6	5.1	4.6	6.2	5.2	<4.0	5.6	4.5	<4.2
- high		- medium molecular mass		- low								

Table 5.3 The different CarbE isoenzymes in lung, liver, plasma and small intestine of rat and guinea pig

Rat									
MAb	Liver				Plasma		Small intestine		
(pI)	6.2	6.0	5.6	5.2	4 4	<4.0	5.4	4.75	<4.2
2H6-1	45	82	36	50	63	<5	45	73	<5
1C1-1	21	52	<5	<5	20	<5	58	<5	<5

Guinea pig												
	Lung			Liver			Plasma			Small intestine		
(pI)	5.9	4.9	<4.2	5.6	5.0	4.6	6.2	5.2	<4.0	5.6	4.5	<4.2
2H6-1	30	<5	<5	<5	<5	<5	73	<5	<5	36	<5	<5
1C1-1	<5	<5	<5	<5	<5	<5	66	30	<5	60	21	<5

Table5. 4 ELISA for reactivity of MAbs (2H6-1 and 1C1-1) with CarbEs
The level of binding of MAbs to rat lung CarbE (pI 5.8) was set to 100%. A reaction of less than 5% was considered negative (Data from Paper III).

Two reports are supporting the idea of a cohesion between the CarbEs in small intestine and plasma: Böcking and Deimling (1982) have proposed that the CarbE isoenzymes participate in the transport of lipids by assuming that one or two of the small intestine CarbE isoenzymes will enter the blood stream with the chylomicrons. Sterri (1989) has also proposed that intestinal CarbE may be a possible source of the enzyme in rat plasma, due to the oxime induced reactivatability and the low pI for the enzyme in both tissues. It has been shown that CarbEs from rat and guinea pig plasma and rat small intestine inhibited by soman can be reativated by diacetylmonooxime, whereas CarbEs from porcine and guinea pig liver are not reactivated (Sterri & Fonnum, 1987, Sterri, 1989; Fonnum *et al.*,1985). Lately Van Lith *et al.* (1992b) have by isoenergetic replacement of carbohydrate by coconut fat raised both serum and small intestinal total CarbE and especially ES-1 activities in rats. They have suggested that ES-1 is involved in the uptake of fatty acid esters by the intestinal brush border membranes.

Others have assumed that CarbE may be synthesized on liver microsomes and secreted to the serum (Little *et al.*,1990). Experiments with thiouracil complemented diet in male and female rat have shown good correlation between AChE activities in liver and plasma, but no such correlation was seen for the CarbE activities in these two tissues (Sterri, unpublished results). These latter results disprove a possible source of the rat plasma CarbEs from the liver CarbEs.

5.5 Heterogeneity of CarbEs in rat liver cells (Paper IV)

In Paper IV, rat liver cells were separated into parenchymal cells (PC), Kupffer cells (KC) and endothelial cells (EC). The distribution of CarbE isoenzymes between these cell types showed that KC and EC contain only those with pI 6.4/6.2, whereas in PC all of the liver CarbE isoenzymes could be detected.

The KC and EC play an important role in the clearance of abnormal or foreign substrates. They also have a specific function in the removal of lipopolysaccharides, cholesterol esters and glycoproteins (Knook & Sleyster, 1980). The liver is the only organ where cholesterol esters can be removed from the circulation and degraded to bile acids (Van Berkel & Van Tol, 1979). What is the function of the CarbE pI 6.4/6.2 in these two cell types? A possible explanation can be that the CarbE pI 6.4/6.2 is acting on the degradation of lipopolysaccharides and/or cholesterol esters. This CarbE isoenzyme has been classified as monoacylglycerol lipase (EC 3.1.1.23) (Mentlein *et al.*,1984b), but also as palmiotyl-CoA hydrolase (EC 3.1.2.2) (Berge, 1979; 1980; Berge & Farstad, 1979; Mentlein *et al.*,1985b). CarbE pI 6.4/6.2 is especially reactive with amphiphilic lipoids of chain length above C_{10} including palmitoyl glycerol and cholesterol oleate (Mentlein *et al.*,1984a). A comparison of the CarbE pI 6.4/6.2 and carboxyl ester lipase (CEL) (EC 3.1.1.3) shows similarities in substrate specificity and in amino acid sequence: CEL has a broad substrate range and is capable of hydrolyzing phospholipids, vitamin esters, cholesterol esters and triacylglycerols. The primary sequence of CEL shows, as CarbEs, an exact conservation of the Gly-Glu-Ser-Ala-Gly sequence near the active site serine (Reue *et al.*,1991).

6 CONCLUSIONS

CarbEs in rat lung have been separated into three isoenzymes with different pI (5.8, 5.5, 5.3). The isoenzyme, pI 5.8, present in highest activity has a molecular mass of approx. 180 kD and with subunits of 60 kDa, which indicate a trimeric structure. This lung CarbE shows a high degree of similarity to the rat liver CarbE, pI 6.0, in molecular mass, N-terminal amino acid sequence and immunochemical relationship.

The lung and liver CarbEs may contribute to the detoxication process in situations where the organophosphorus compounds have higher affinities for CarbE than for AChE, which means the less toxic compounds like paraoxon and DFP.

Immunochemical characterization using monoclonal antibodies indicates that at least two distinct categories of CarbE isoenzymes are present in plasma as well as small intestine, and that intestinal CarbE may be the source of the plasma enzyme in both rat and guinea pig.

The Kupffer cells and endothelial cells of the rat liver contain only the CarbE pI 6.2/6.4, whereas in the parenchymal cells all the known liver CarbE isoenzymes can be detected.

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ERRATA

In the papers, the following errors should be corrected to:

Paper I, References, line 2 from below: *Biochem Pharmacol* **32**, 2779-2785.

Paper II, Fig. 7. line 2: " after paraoxon injection".

Paper III, Reference 5: *Nature* **256,** 495-497, 1975.

Paper I

Biochem. J. (1991) 274, 693-697 (Printed in Great Britain)

Purification and characterization of carboxylesterases from rat lung

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Carboxylesterase (EC 3.1.1.1) has played an important part in our understanding of the toxicokinetic behaviour of the organophosphorus cholinesterase inhibitors. Carboxylesterases are a heterogeneous group of enzymes that can be separated on the basis of their isoelectric points and by their substrate-specificity. We have purified the isoenzyme (pI 5.8) present in greatest activity in rat lung to near homogeneity. The enzyme was purified by $(NH_4)_2SO_4$ precipitation, gel filtration, chromatofocusing, separation on anion- and cation-exchangers and hydrophobic-interaction chromatography. The purified enzyme has a molecular mass of approx. 180 kDa with subunits of 60 kDa. The substrate and inhibitor specificities of the enzyme have been characterized. Edman degradation revealed the first 19 amino acid residues of the enzyme. The *N*-terminus was found to be tyrosine. Inhibition of the enzyme by organophosphorus compounds differed greatly from that of cholinesterases, despite the partial analogy at the *N*-terminal region.

INTRODUCTION

Carboxylesterases (EC 3.1.1.1) have been shown to play an important part in the detoxification of organophosphorus compounds (Sterri & Fonnum, 1984; Fonnum *et al.*, 1985). They are a heterogeneous group of enzymes that differ in isoelectric point and in substrate-specificity. Most investigations have been conducted with the esterases of rat liver. In the last years, six carboxylesterases have been isolated from rat liver microsomal fractions (Mentlein *et al.*, 1980; Robbi & Beaufay, 1983). However, all the esterases examined probably exist in microheterogeneous forms.

The isoelectric points and molecular masses of the liver microsomal carboxylesterases have been determined, but only short fragments of the active-site and the *N*- and *C*-terminal amino acid sequences are known (Heymann *et al.*, 1970; Mentlein *et al.*, 1984). The isoelectric points and the substrate specificities of the carboxylesterases from other tissues have in part been determined, but very little knowledge exists with regard to their purification.

In the present investigation we wanted to characterize carboxylesterases from rat lung, which is one of the main tissues where they can be found. We report on the purification and characterization of the most abundant isoenzyme from this tissue.

MATERIALS AND METHODS

Chemicals

4-Nitrophenyl butyrate, 4-nitrophenyl acetate, α-naphthyl acetate and bis-(4-nitrophenyl) phosphate were from Sigma Chemical Co., St. Louis, MO, U.S.A. Butanilicaine (*N*-butyl-aminoacetyl-2-chloro-6-methylanilide) was from Hoechst A.G., Frankfurt, Germany. Sephadex G-25 (medium grade), Ultrogel AcA34, Polybuffer exchanger 94, Polybuffer 74, Mono Q HR 5/5, Mono S HR 5/5 and alkyl-Superose HR 5/5 were from Pharmacia LKB Biotechnology Division, Uppsala, Sweden. Paraoxon (diethyl 4-nitrophenyl phosphate) was from Koch-Light Laboratories, Colnbrook, Berks., U.K. Di-isopropyl phosphorofluoridate was from Du Pont NEN Research Products, Dreieich, Germany. Soman (pinacolyl methylphosphonofluoridate), sarin (isopropyl methylphosphonofluoridate), cyclosarin (cyclohexyl methylphosphonofluoridate) and tabun (*O*-ethyl *NN*-dimethylphosphoramidocyanidate) were synthesized in this laboratory by the procedures of Bryant *et al.* (1960) and Holmstedt (1951), and the purity was found to be greater than 95% by n.m.r. spectroscopy. All other chemicals were of analytical-grade quality.

Animals

Male Wistar rats (200–300 g weight) were purchased from the National Institute of Public Health, Oslo, Norway.

Purification procedures

Lungs were collected from male Wistar rats (200–300 g), which were macroscopically free from disease. A 40–70 g batch of the tissue was rinsed with cold 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl and homogenized in 4 vol. of the same buffer with a Polytron instrument for 20 s. The homogenate was filtered through glass-wool to remove fat, and heated at 55 °C for 15 min to remove unstable proteins, followed by centrifugation at 9000 *g* for 20 min at 4 °C. The supernatant was further centrifuged at 100000 *g* for 60 min. The high-speed supernatant was fractionated by $(NH_4)_2SO_4$ precipitation. The 35–70%-satd.-$(NH_4)_2SO_4$ fraction was resuspended in a small volume of 50 mM-Tris/HCl buffer, pH 8.0.

Gel filtration. The 35–70%-satd.-$(NH_4)_2SO_4$ fraction was applied to an Ultrogel AcA34 gel-filtration column (60 cm × 2.5 cm) at 4 °C. The column was equilibrated and eluted with 50 mM-Tris/HCl buffer, pH 8.0. The flow rate was 0.35 ml/min, and 4 ml fractions were collected. Fractions containing 80–90% of the total activity were combined and concentrated to 5–6 ml by means of an Amicon ultrafiltration cell, with a YM-10 membrane.

Chromatofocusing. The preparation was chromatofocused at 4 °C as described in the chromatofocusing kit instructions (Pharmacia LKB). The sample buffer was exchanged with the

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starting buffer 25 mM-imidazole/HCl buffer, pH 7.4, on a Pharmacia PD-10 column. The elution buffer was an 8-fold dilution of Polybuffer 74, adjusted to pH 4.0 with 1.0 M-HCl. A column (0.9 cm × 28 cm) was packed with PBE 94 and equilibrated with the starting buffer. The sample was applied after running through 5 ml of the elution buffer. In all, 200 ml of elution buffer was followed by 2 column vol. of 1 M-NaCl solution. The elution rate was 0.40 ml/min, and 2 ml fractions were collected. Fractions containing 80% of the total activity were combined and concentrated to 6 ml with an Amicon ultrafiltration cell.

The chromatofocusing procedure was repeated, but with other buffers. The same column (PBE 94) was equilibrated with the starting buffer 25 mM-histidine/HCl buffer, pH 6.2, and the elution buffer was a 10-fold dilution of Polybuffer 74/HCl, pH 5.0. Fractions with the highest activity (fraction A) were combined and concentrated to 2.5 ml with an Amicon ultrafiltration cell.

Anion-exchange chromatography (f.p.l.c.) on Mono Q HR 5/5. The buffer of fraction A (pI 5.8) was exchanged with the starting buffer 25 mM-Bistris/HCl buffer, pH 7.0. The material was applied to a Mono Q HR 5/5 column, which was eluted (1.0 ml/min) with 6 ml of the starting buffer followed by 20 ml of a linear gradient of 0–0.5 M-NaCl in the starting buffer.

Cation-exchange chromatography (f.p.l.c.) on Mono S HR 5/5. The buffer of the combined fractions was exchanged with the next starting buffer 25 mM-Mes/NaOH buffer, pH 5.1. The preparation was applied to a Mono S HR 5/5 column, which was eluted (1.0 ml/min) with 6 ml of the starting buffer followed by 20 ml of a linear gradient of 0–0.5 M-NaCl in the starting buffer.

Hydrophobic interaction (f.p.l.c.) on alkyl-Superose HR 5/5. The buffer of the collected fractions was exchanged with 50 mM-sodium phosphate buffer, pH 7.0, containing 1.5 M-$(NH_4)_2SO_4$. The preparation was applied to alkyl-Superose HR 5/5 equilibrated with 50 mM-sodium phosphate buffer, pH 7.0, containing 2.0 M-$(NH_4)_2SO_4$. The column was eluted (0.5 ml/min) with a linear gradient of 2.0–0 M-$(NH_4)_2SO_4$ in the starting buffer. Fractions with the highest activity were combined, and the buffer was exchanged with 25 mM-ammonium bicarbonate buffer, pH 8.0. The protein solution was concentrated by drying under vacuum.

Enzyme assay methods

Carboxylesterase activity was measured spectrophotometrically with 4-nitrophenyl butyrate as substrate (Sterri *et al.*, 1985). The assay mixture at 30 °C consisted of 0.1 M-sodium phosphate buffer, pH 7.8, 2 mM-4-nitrophenyl butyrate and tissue in a total volume of 3.02 ml. The absorbance of 4-nitrophenol was monitored at 400 nm with a Beckman DU-50 spectrophotometer. The molar absorption coefficient for 4-nitrophenol at pH 7.8, used in this investigation, was 17000 $M^{-1} \cdot cm^{-1}$.

The substrate-specificity of rat lung carboxylesterase was also examined with 4-nitrophenyl acetate and butanilicaine. 4-Nitrophenyl acetate was used under the same absorption coefficient. When butanilicaine was used, the assay mixture consisted of 0.06–5.00 mM substrate and 50 mM-Tris/HCl buffer, pH 8.6, as described by Heymann *et al.* (1981). The increase in absorbance was monitored at 285 nm, and a molar absorption coefficient of 2080 $M^{-1} \cdot cm^{-1}$ was used.

Measurement of inhibition

The purified enzyme was incubated with different organophosphates in 0.1 M-sodium phosphate buffer, pH 7.8, at 30 °C. After various times (5–20 min) the substrate, 4-nitrophenyl butyrate, was added and the residual activity determined as described above.

The bimolecular rate constants were calculated from the equation:

$$k_i = [\ln(100 - v)]/(t \cdot c)$$

where c is the concentration of inhibitor, v is the percentage of esterase activity remaining and t is the time of preincubation of enzyme and inhibitor before the addition of substrate (Aldridge & Reiner, 1972).

SDS/PAGE

SDS/PAGE was carried out as described by Laemmli (1970), on the Phast Gel System from Pharmacia. A polyacrylamide-gradient gel, Phast Gel Gradient 8–25, was used with Phast Gel SDS buffer strips.

Determination of molecular mass

Molecular mass was determined by the gel-sieving method of Johnson (1979). The PAGE was run with a Mini Protean II cell from Bio-Rad Laboratories. Different concentrations of acrylamide (4, 7 and 10%) were used. The standard proteins used were the high-molecular-mass standards of Bio-Rad Laboratories. The protein was located in the gels by using Coomassie Brilliant Blue or the substrate α-naphthyl acetate. The gels were run and stained by the method of Oien & Stenersen (1984).

Amino acid sequence analyses

Sequence analysis was carried out on an Applied Biosystems 477A Protein Sequencer. The amino acid phenylthiohydantoin derivatives were determined on-line with the aid of an Applied Biosystems 120 phenylthiohydantoin analyser, as outlined by Cornwell *et al.* (1988).

Other analytical methods

Protein was determined as described by Lowry *et al.* (1951), with BSA as standard.

RESULTS

In rat lung the highest carboxylesterase activity (more than 80% of total activity) was found in the 100000 *g* supernatant, with 4-nitrophenyl butyrate as substrate. The yield of solubilized

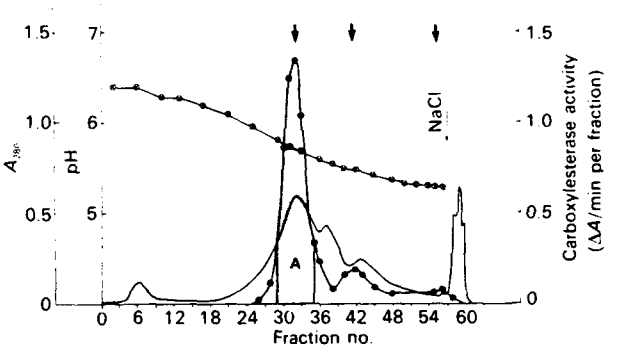


Fig. 1. Chromatofocusing II of rat lung preparation

The eluted fractions were analysed for carboxylesterase activity (●), pH (⊗) and A_{280} (——). The column was eluted with 1 M-NaCl after the fraction indicated by an arrow. The thick arrows indicate the three isoelectric points (pI). The hatched peak contained the fraction with the highest hydrolytic activity on 4-nitrophenyl butyrate, and the fraction (A) was used in the next purification step.

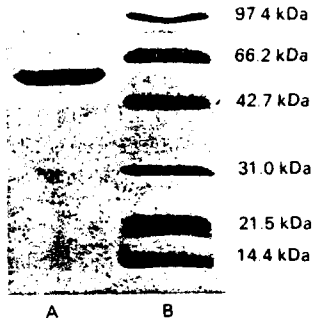


Fig. 2. SDS/PAGE of purified carboxylesterase (pI 5.8)

The electrophoresed gel was stained with Coomassie Brilliant Blue R-250. Lane A, 4.5 μg of protein. Lane B, molecular-mass markers (phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and lysozyme in order of decreasing molecular mass).

enzyme did not increase after use of the glycoside detergent saponin (final concn. 1%, w/w) to extract the microsomal activity. The rest of the activity or the microsomal activity was distributed in various subcellular fractions (present in the 600 *g*, 6500 *g*, 12000 *g* and 100000 *g* pellets).

Carboxylesterase was separated into different isoenzymes according to their isoelectric points (pI) by chromatofocusing. Fig. 1 show three different isoenzymes, pI 5.8, pI 5.5 and pI 5.3. The isoenzyme present in greatest activity, pI 5.8, was purified by f.p.l.c. to a single band on SDS/PAGE with a monomer mass of 60 kDa (Fig. 2). The molecular mass was determined to be 180 kDa by the gel-sieving method. Because the protein appeared to be essentially homogeneous, we have to assume a trimeric structure of identical subunits. In the purification experiment reported in Table 1, the yield of the enzyme was 1.0 mg of protein/70 g of tissue. The low enzyme recovery was in part due to the fact that only one of the isoenzymes was selectively purified.

Substrate-specificity

The results on the substrate-specificity of rat lung carboxylesterase (pI 5.8) are given in Table 2. The activities were determined at various substrate concentrations, and maximal velocity, V, and the Michaelis constant, K_m, were determined graphically from Lineweaver–Burk plots.

4-Nitrophenyl acetate and 4-nitrophenyl butyrate are examples of esters with an aromatic alcohol group. Both substrates are rapidly hydrolysed by rat lung carboxylesterase, with the highest V and K_m for 4-nitrophenyl acetate. Substrate inhibition was observed at high concentrations (> 1.6 mM) for both substrates.

Butanilicaine, a representative of the aromatic amides, is hydrolysed at a much lower rate than the esters. This is typical for carboxylesterases.

Inhibition by organophosphorus compounds

The rates of inactivation of different esterases, including carboxylesterase (pI 5.8), by organophosphorus compounds are shown in Table 3. The bimolecular rate constant of inhibition was determined from a number of experiments with different concentrations and incubation times. The inactivation reaction followed first-order kinetics, since the decrease in log(activity) was linear with time for several concentrations of inhibitors. The inactivation reaction follows first-order kinetics when $c_i/[E_0] \geqslant 10$ (Aldridge & Reiner, 1972), where $[E_0]$ and c_i are initial concentrations of enzyme and inhibitor. In our experiments the maximal value of $[E_0]$, estimated by assuming pure enzyme, varied from 0.3 to 1.6 nM. Whereas acetylcholinesterase showed wide variations in specificity towards inhibitors, the carboxylesterases gave inhibitor rate constants in a narrow range. Bis-(4-nitrophenyl) phosphate is a very poor inhibitor of

Table 1. Purification of carboxylesterase (pI 5.8) from rat lung

For experimental details see the text.

Fraction	Volume (ml)	Protein (mg)	Specific activity (μmol/h per mg)	Purification (fold)	Recovery (%)
I Protein extract	350	8000	17	1	100
II 35–70%-satd.-$(NH_4)_2SO_4$ fraction	30	960	60	4	42
III Gel filtration on Ultrogel AcA34	7.3	201	168	10	25
IV Chromatofocusing I	6.0	38	297	18	8
V Chromatofocusing II	1.2	11	920	54	7
VI Anion-exchange chromatography on Mono Q HR 5/5 and cation-exchange chromatography on Mono S HR 5/5	2.0	1.7	3750	221	5
VII Hydrophobic-interaction chromatography on alkyl-Superose HR 5/5	0.3	1.0	5650	332	4

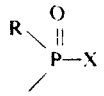
Table 2. Kinetics of the hydrolysis of esters and amides by carboxylesterase (pI 5.8)

The enzyme concentration ($[E_0]$) was 3.0 nM. Results are means ± S.E.M. ($n = 4$).

Substrate	Range of substrate concentration (mM)	K_m (mM)	V (μmol/min per mg)
4-Nitrophenyl acetate	0.5–0.08	0.06 ± 0.01	107 ± 4
4-Nitrophenyl butyrate	0.6–0.05	0.02 ± 0.005	64 ± 3
Butanilicaine	5.0–0.06	0.43 ± 0.01	15 ± 2

Table 3. Bimolecular rate constants for the reaction of rat lung carboxylesterase (pI 5.8), horse liver carboxylesterase and bovine erythrocyte acetylcholinesterase with organophosphorus compounds

Results for rat lung carboxylesterase are means ± S.E.M. (n = 6–9). Values for horse liver carboxylesterase and bovine erythrocyte acetylcholinesterase are taken from Ooms (1961).

Organophosphorus compound General formula 				k_i (M^{-1} min^{-1})		
				Carboxylesterase from:		Acetylcholinesterase from bovine erythrocytes
	X	R	R'	Rat lung	Horse liver	
Bis-(4-nitrophenyl) phosphate	$OC_6H_4NO_2$	$OC_6H_4NO_2$	O	$(1.8 \pm 0.3) \times 10^5$		
Paraoxon	$OC_6H_4NO_2$	OC_2H_5	OC_2H_5	$(1.4 \pm 0.2) \times 10^7$	7.9×10^6	4.5×10^7
Soman	F	CH_3	$OCH(CH_3)C(CH_3)_2$	$(7.0 \pm 0.6) \times 10^6$	2.3×10^5	6.0×10^7
Sarin	F	CH_3	$OCH(CH_3)_2$	$(6.0 \pm 0.2) \times 10^5$	1.7×10^5	1.4×10^7
Cyclosarin	F	CH_3	OC_6H_{11}	$(2.2 \pm 0.4) \times 10^6$	5.3×10^5	3.3×10^7
Di-isopropyl phosphoro-fluoridate	F	$OCH(CH_3)_2$	$OCH(CH_3)_2$	$(2.8 \pm 0.3) \times 10^6$	3.2×10^5	4.6×10^4
Tabun	CN	OC_2H_5	$N(CH_3)_2$	$(3.6 \pm 0.4) \times 10^6$	7.0×10^5	3.6×10^6

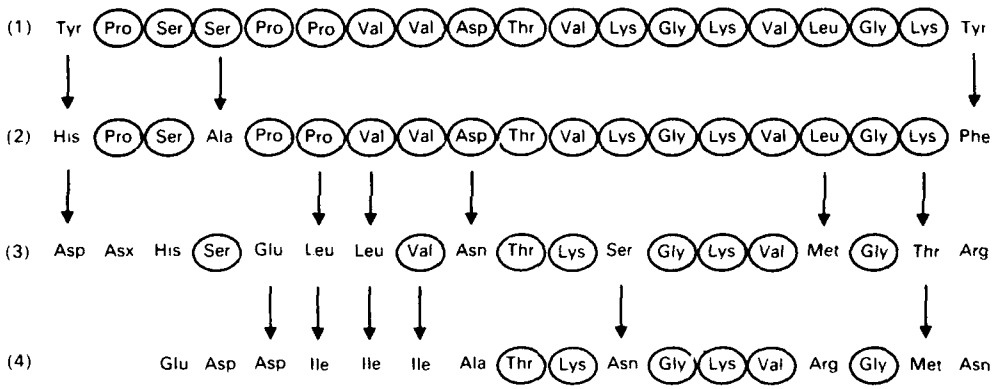


Fig. 3. Sequence comparison of the *N*-terminal regions of (1) rat lung carboxylesterase (pI 5.8), (2) 60 kDa rabbit liver microsomal esterase, (3) *Torpedo* acetylcholinesterase and (4) human butyrylcholinesterase

The ringed amino acids indicate identical sequences between (1), (2), (3) and (4). Arrows indicate functionally related amino acids and codons that differ by a single nucleotide base. The 60 kDa rabbit liver microsomal esterase sequence was taken from Korza & Orzols (1988), the acetylcholinesterase sequence from MacPhee-Quigley *et al.* (1985) and the butyrylcholinesterase sequence from Lockridge *et al.* (1987).

acetylcholinesterase (the bimolecular rate constant is not shown). The data for rat lung are strikingly higher than those previously published for horse liver (Ooms, 1961). It was also shown that physostigmine, at a concentration of 1 mM, did not inhibit carboxylesterase (pI 5.8).

N-Terminal amino acid sequence

The *N*-terminal sequence of the rat lung carboxylesterase shows partial analogy with the *N*-terminal sequence of *Torpedo* acetylcholinesterase and slightly lower analogy with human serum butyrylcholinesterase. The lung carboxylesterase sequence shows a high degree of similarity to a recently published microsomal esterase from rabbit liver (Fig. 3). Of the esterases compared, only butyrylcholinesterase has a glycosylation site within the 19 residues of the *N*-terminal sequence.

DISCUSSION

The carboxylesterase activity of rat lung (measured with the substrate 4-nitrophenyl butyrate) was mainly found in the cytosolic fraction, and there was no advantage in extracting the small amount of microsomal activity. Also, malathion carboxylesterase from lung has been shown to be localized predominantly in the 100000 *g* supernatant, the cytosolic fraction (Ali & Imamura, 1985). In contrast, malathion carboxylesterase activity in liver was found to be mainly localized (75%) in the microsomal fraction (Talcott, 1979). These results show that the subcellular pattern of carboxylesterases in the lung is different from that in liver. The most likely explanation is that the enzyme is localized almost exclusively in the soluble fraction of the lung, but autolysis, which readily solubilizes the membrane-bound carboxylesterase, cannot be excluded.

There are no previous reports on the purification of rat lung carboxylesterases, but the isoelectric points have previously been measured. The isoelectric points found here for the two peaks of carboxylesterase activity in lung, pI 5.8 and pI 5.5 (see Fig. 1), are close to those reported by Mentlein *et al.* (1987) (pI 6.0 and pI 5.6). The small differences observed could be due either to the fact that two different outbred strains of Wistar rats were used or to the fact that isoelectric focusing was used by Mentlein *et al.* (1987) whereas we used chromatofocusing. Experiments have shown that the pI may be slightly changed by repeated isoelectric focusing (Heymann & Junge, 1979) and also by chromatofocusing in ampholytes (Goeppinger *et al.*, 1978).

The isoenzyme (pI 5.8) was shown by SDS/PAGE to have a subunit mass of about 60 kDa, but a molecular mass of 180 kDa was obtained by the gel-sieving method. It is therefore reasonable to assume a trimeric structure and that each subunit has one active site. The lung isoenzyme therefore bears some resemblance to the rat liver carboxylesterase (pI 6.0), which is also a trimer (Mentlein *et al.*, 1984). A trimeric structure has also been reported for a pig liver carboxylesterase (Heymann & Junge, 1979).

The length of the alkyl and alkoxy groups seems to be important for the inhibitory properties of organophosphorus compounds. An analogous effect is found with substrates: the rate of hydrolysis depends on the number of carbon atoms in the acyl group (Aldridge & Reiner, 1972). Considering the two substrates 4-nitrophenyl acetate and 4-nitrophenyl butyrate (Table 2), lengthening the substrate by two carbon atoms nearly doubles the rate constant (V/K_m). The pattern for the inhibitors is more difficult to explain (Table 3). The rate constant for the carboxylesterase inactivation is increased when the alkoxy group is lengthened. Inhibition of acetylcholinesterase also increases with the length of the alkoxy group of the organophosphorus compound, but it differs from carboxylesterase in that two bulky alkoxy groups showed the lowest rate of inactivation (Table 3). A striking difference between the two enzymes is the fact that acetylcholinesterase is not inhibited by bis-(4-nitrophenyl) phosphate. The negative charge on the inhibitor probably prevents binding to the esteratic locus of the acetylcholinesterase active site because of the nearby anionic locus. Carboxylesterase (pI 5.8) differs further from cholinesterases in that it is not inhibited by physostigmine at a concentration of 1 mM. We therefore exclude any relationship to the cholinesterases, despite the sequence similarities at the *N*-terminal region.

In general, good inhibitors are found to be compounds that resemble the structure of substrates. An example is paraoxon, the best inhibitor of the organophosphorus compounds examined, the structure of which resembles that of 4-nitrophenyl butyrate.

Soman and sarin have rate constants of inactivation of carboxylesterase (pI 5.8) that approach the rate constants of inhibition of acetylcholinesterase (Table 3). These results explain how carboxylesterase could function as a scavenger in soman detoxification (Fonnum *et al.*, 1985).

The six rat liver carboxylesterase isoenzymes have different *N*-terminal amino acid residues, which are tyrosine and aspartic acid/asparagine (Mentlein *et al.*, 1984), but the structural analyses of these preparations have yet to be performed. The *N*-terminal residue of carboxylesterases from other species such as pig liver was shown to be glycine, but no sequence has been determined (Heymann & Iglesia, 1974). These results show that the isoenzymes separated from various animals and tissues with a subunit mass of approx. 60 kDa have differences in the *N*-terminus and can illustrate heterogeneity of the carboxylesterases.

The recently presented amino acid sequence of the 60 kDa glycoprotein esterase from rabbit liver microsomal fractions (Korza & Ozols, 1988) shows a high degree of similarity to the reported *N*-terminal sequence of carboxylesterase (pI 5.8). The differences observed are in positions 1, 4 and 19 (Fig. 3), and we have to assume a close relationship between these two enzymes.

The present paper reports the first amino acid sequence of a rat carboxylesterase, and the data could give further information on the relationship between the different carboxylesterases, and on the understanding of physiological detoxification.

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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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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

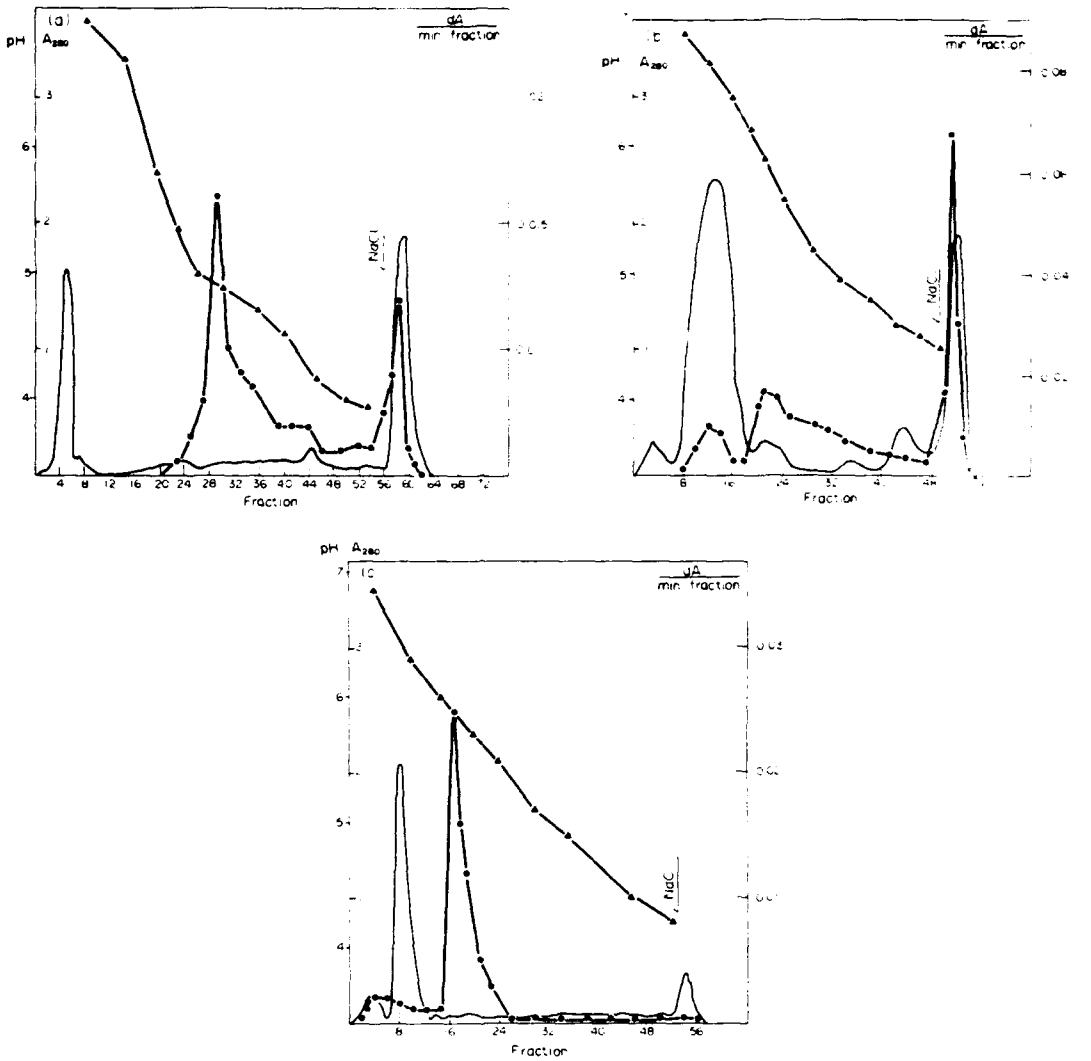

Fig. 1. Chromatofocusing of guinea pig lung after gel filtration. (a) high molecular mass preparation. (b) medium molecular mass preparation, (c) low molecular mass preparation. The eluted fractions were analysed for CarbE activity: dA/min·fraction (●), pH (▲) and A_{280} (−). The column was eluted with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

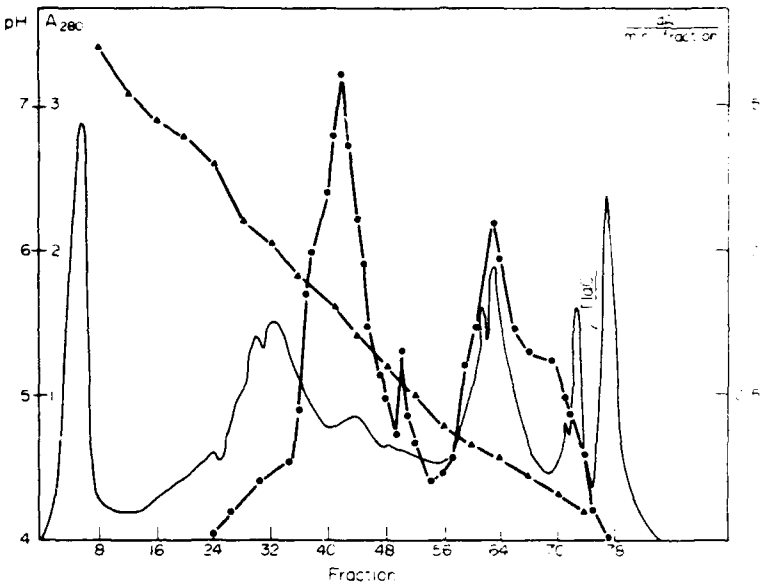

Fig. 2. Chromatofocusing of guinea pig liver preparation. The eluted fractions were analysed for CarbE activity: dA/min·fraction (●), pH (▲) and A_{280} (−). The column was eluted with 1 M sodium chloride solution after the fraction indicated by an arrow.

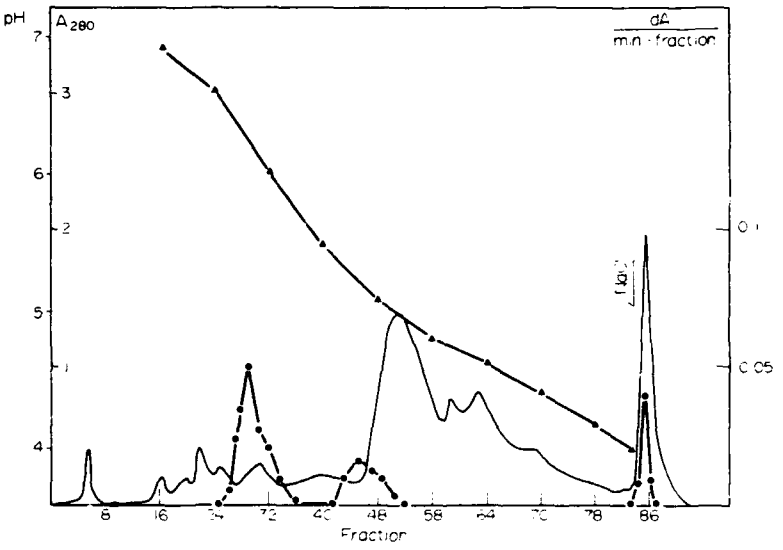

Fig. 3. Chromatofocusing of guinea pig plasma preparation. The eluted fractions were analysed for CarbE activity: dA/min·fraction (●), pH (▲) and A_{280} (−). The column was eluted with 1 M sodium chloride solution after the fraction indicated by an arrow.

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).

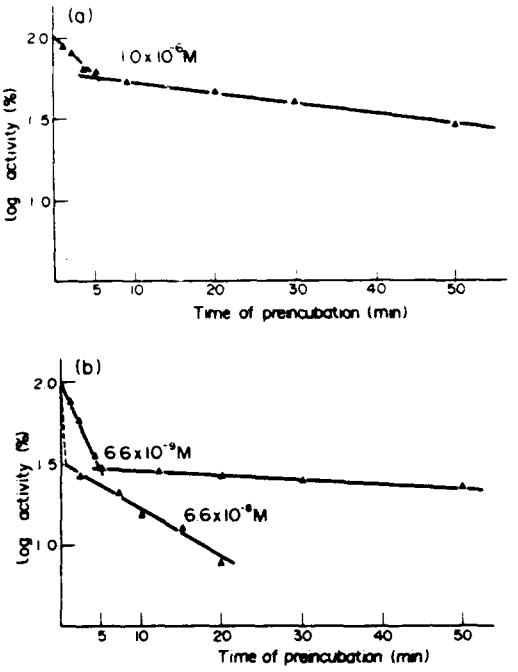

Fig. 4. The rate of inactivation of CarbE, pI < 4.2, from guinea pig lung by (a) bis(*p*-nitrophenyl)phosphate (1.0×10^{-6} M) and (b) paraoxon (6.6×10^{-9} M and 6.6×10^{-8} M). Residual activity was measured after preincubation with the different concentrations of inhibitor.

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

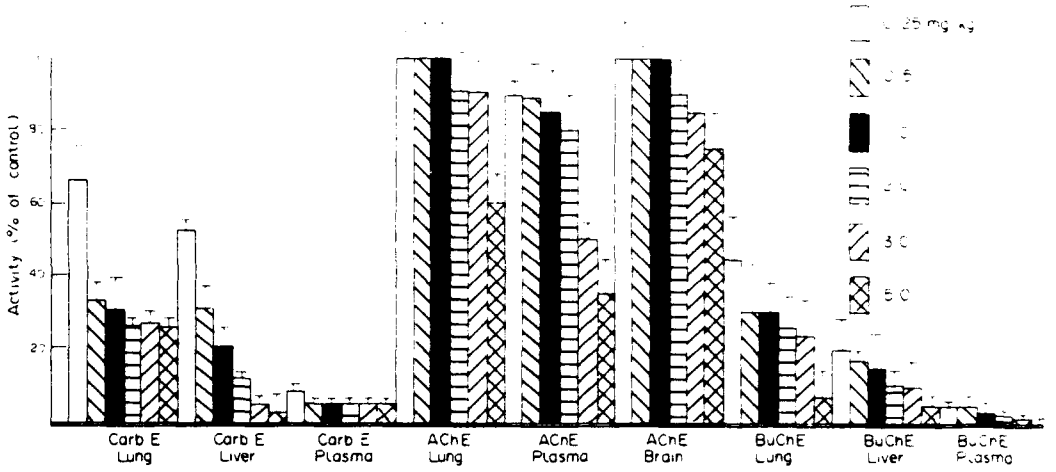

Fig. 5. The effect of intraperitoneal administration of DFP on CarbE, AChE and BuChE activities in guinea pig. (Animals taken for analysis 1.5 hr after DFP injection of 0.25–5.0 mg/kg i.p.) Error bars show SDs of values obtained in assays from four separate animals.

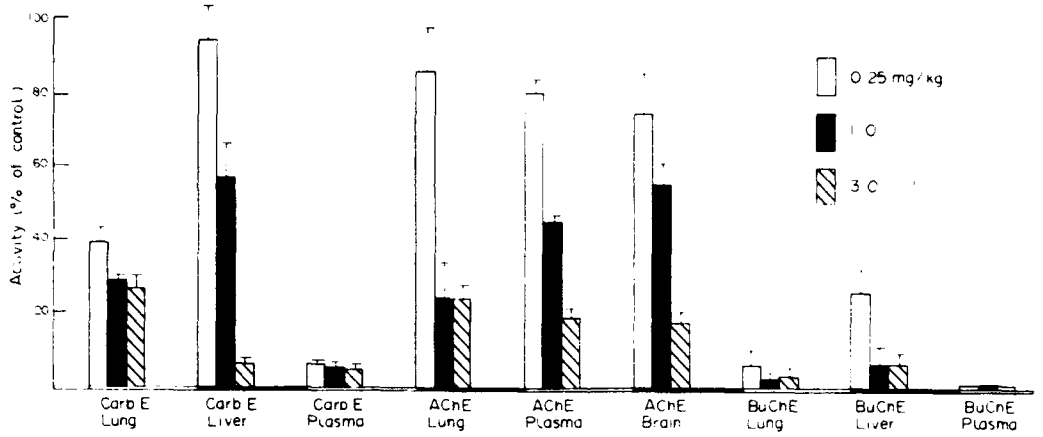

Fig. 6. The effect of subcutaneous administration of DFP treatment on CarbE, AChE and BuChE activities in guinea pig. (Animals taken for analysis 1.5 hr after DFP injection of 0.25–3.0 mg/kg s.c.) Error bars show SDs of values obtained in assays from four separate animals.

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

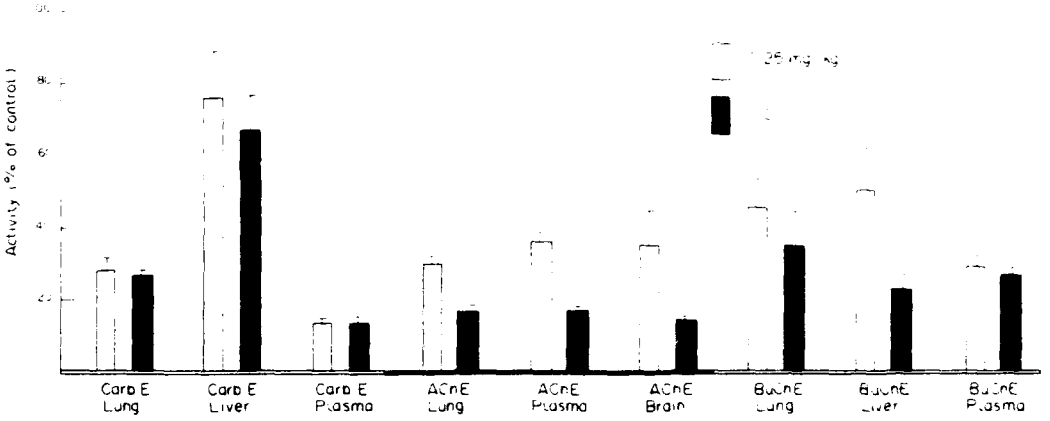

Fig. 7. The effect of subcutaneous administration of paraoxon on CarbE, AChE and BuChE activities in guinea pig. (Animals taken for analysis 1.5 hr after DFP injection of 0.25–1.0 mg/kg s.c.) Error bars show SDs of values obtained in assays from four separate animals.

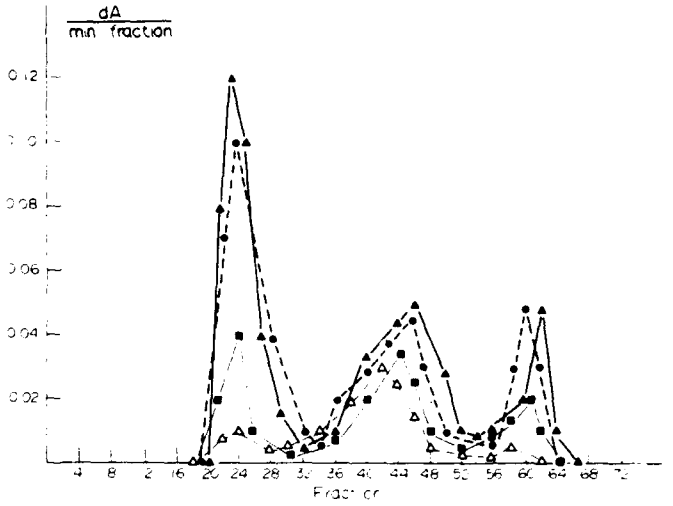

Fig. 8. Representative gel filtration profiles of lung CarbE activity in guinea pig treated *in vivo* with DFP 0.25 mg/kg i.p. (●), 0.5 mg/kg i.p. (■) or with paraoxon s.c. (△). Gel filtration of lung homogenate without any treatment (▲)

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.

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Paper III

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SHORT COMMUNICATIONS

Monoclonal antibodies distinguish between carboxylesterase isoenzymes in different tissues of rat and guinea pig

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Abstract—The carboxylesterase (CarbE) activity in the main tissues (lung, liver, plasma and small intestine) of both the rat and guinea pig was separated by chromatofocusing. The three CarbE isoenzymes in the small intestine from both species showed nearly identical pI values. Monoclonal antibodies (MAbs) raised against rat lung CarbE (pI 5.8) were used in enzyme-linked immunosorbent assays to distinguish between these closely related CarbE isoenzymes. None of the MAbs did bind to the active site as no inhibition of the enzyme was seen when the MAbs were added. The immunological study showed a strong relationship between lung CarbE (pI 5.8) and the rat liver CarbE (pI 6.0). The MAbs were also strongly bound to the high pI forms of the CarbE isoenzymes in plasma and small intestine from both rat and guinea pig, but not with the low pI forms. These results indicate that two immunochemically distinct categories of CarbE isoenzymes are present in the plasma and small intestine.

Carboxylesterases (CarbEs*) (EC 3.1.1.1) are a group of B-esterases which are characterized by a broad substrate specificity for aliphatic and aromatic esters as well as for aromatic amides. They are important in the hydrolytic transformation of many toxic pesticides, insecticides and drugs, and in the detoxification of organophosphorus compounds by covalent binding to the active sites of the enzymes. The unusual broad substrate specificity of the CarbEs is due, in part, to multiple CarbE isoenzymes. Many of these isoenzymes have now been partially purified, but the number of forms and the diversity of their structure are only beginning to be clarified.

Immunochemical approaches with polyclonal antisera have previously been used to reveal the distinct character of the liver CarbE isoenzymes [1], but the specificity of monoclonal antibodies might be more ideally suited to being used as probes of the structure and function of different forms of CarbE. In the present study we have raised murine monoclonal antibodies (MAbs) to rat lung CarbE isoenzyme (pI 5.8) by the hybridoma technology, and these MAbs have been used in enzyme-linked immunosorbent assays (ELISA) to distinguish between the different CarbE isoenzymes in the tissues (lung, liver, plasma and small intestine) of both the rat and guinea pig.

Materials and Methods

Chemicals. Sephadex G-25M, Ultrogel AcA 34, Polybuffer exchanger 94, Polybuffer 74, MAb trap G kit with a Protein G Sepharose 4 fast flow column were from Pharmacia LKB, Biotechnology Division (Uppsala, Sweden). 4-Nitrorophenyl butyrate, Freund's Complete and Incomplete Adjuvants, polyethylene glycol (M_r 1300–1600), 2,2'-azinodi-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ATBS) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Mouse monoclonal antibody isotyping kit was from Amersham International (Amersham, U.K.). Nunc Immuno plates were from Nunc (Roskilde, Denmark). Biotin-labeled goat anti-mouse IgG and horseradish peroxidase-labeled streptavidin were from Southern Biotechnology Associates (Birmingham, U.S.A.). Fetal bovine serum was from Hyclone Laboratories (Logan, U.S.A.). NSO/1 myeloma cells were kindly provided by Professor Z. Eshar, Weissman Institute, Rehovot, Israel. All other chemicals were of analytical-grade quality.

Animals. Male Wistar rats (200–300 g weight), guinea pigs (male albino within the weight range 200–400 g) and Balb/c mice (15–20 g weight) (from Møllegaard, Copenhagen, Denmark) were examined at the National Institute of Public Health, Oslo, Norway. The animals were given a standard laboratory diet and water *ad lib.*

Preparation of rat and guinea pig tissue fractions. Rat or guinea pig small intestine was rinsed with ice-cold 50 mM Tris, pH 7.5, containing 0.1 M NaCl and homogenized in 4 vol. of the same buffer with a Polytron instrument for 20 sec. The homogenate was filtered through glass wool to remove fat, followed by centrifugation at 10,000 *g* for 20 min at 4°. The sample buffer was exchanged with the starting buffer, 25 mM imidazole–HCl buffer, pH 7.4, on a Pharmacia PD-10 column. The preparation was chromatofocused at 4° as described in the chromatofocusing kit instruction (Pharmacia LKB). Rat and guinea pig liver, lung and plasma fractions were obtained as described previously [2].

Enzyme assay method. CarbE activity was measured spectrophotometrically with a Beckman DU-50 spectrophotometer and 4-nitrophenyl butyrate was used as substrate [3].

The rat lung CarbE (pI 5.8) was incubated with the different MAbs in 0.1 M sodium phosphate buffer, pH 7.8, 30° at molar ratios of IgG ($M_r \approx 160{,}000$):CarbE (pI 5.8, $M_r \approx 180{,}000$) of about 0.25, 1 and 6. After various times (5–20 min), the substrate, 4-nitrophenyl butyrate, was added (final concn 2 mM) and the residual activity determined as described above, but with a total volume of 0.2 mL. The absorbance of 4-nitrophenol at 400 nm was now followed with a Dynatech MR 700.

SDS–PAGE. SDS–PAGE was performed according to Laemmli [4] on the Phast Gel System from Pharmacia LKB. A polyacrylamide gradient gel, Phast Gel Gradient 8-25, was used with Phast Gel SDS buffer strips.

Preparation of anti-CarbE MAbs. MAbs were produced by the method described by Köhler and Milstein [5]. One of the carboxylesterase isoenzymes (pI 5.8) purified from rat lung was used in the MAb production [6].

The frozen positive subclones were thawed and transferred to growth medium. For large scale production of MAbs *in vitro* both low (2%) and high (10%) serum concentrations were used. MAbs were purified from culture supernatant by using affinity chromatography. A MAb trap G kit with a Protein G Sepharose column from Pharmacia LKB was used. The cell culture supernatant (~25 mL) was

* Abbreviations: CarbE, carboxylesterase; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assays; PBS, phosphate-buffered saline.

Table 1. ELISA for reactivity of MAbs with CarbEs

MAb	Ig subclass	Rat (pI)									Guinea pig (pI)								
		Liver			Plasma		Small intestine		Lung		Liver			Plasma		Small intestine			
		6.2	6.0	5.6	5.2	4.4	<4.0	5.4	4.75	<4.2	5.9	4.9	<4.2	5.6	5.0	4.6	6.2	5.2	<4.0	5.6	4.5	<4.2
2H6-1	IgG1, k	45 ± 8	82 ± 7	36 ± 5	50 ± 8	63 ± 5	<5	45 ± 7	73 ± 10	<5	30 ± 8	<5	<5	<5	<5	<5	73 ± 3	<5	<5	36 ± 5	<5	<5
1C1-1	IgG1, k	21 ± 8	52 ± 2	<5	<5	20 ± 7	<5	58 ± 9	<5	<5	<5	<5	<5	<5	<5	<5	66 ± 8	30 ± 5	<5	60 ± 6	21 ± 4	<5

Results are percentages of control, means ± SEM (N = 4).
The ELISA conditions for testing the binding of MAbs to CarbE isoenzymes are described in the text. The level of binding of MAbs to rat lung CarbE (pI 5.8) was set to 100%. A reaction of less than 5% was considered negative.

applied to the top fit and absorbed into the gel. Unbound protein was washed away with 20 mM sodium phosphate buffer, pH 7.0. A neutralizing buffer (1.0 M Tris–HCl pH 9) was added to the collection tubes and IgG was eluted with a 0.1 M glysin–HCl buffer, pH 2.7.

ELISA. ELISA was used in these studies to analyse for antibody production and in the experiments analysing for antibody binding to CarbE isoenzymes from different tissues. Antigens to be tested against MAbs were diluted and titrated in phosphate-buffered saline (PBS) buffer (pH 7.4), and incubated overnight at 20° in Nunc Immuno plates. Plates were washed three times with PBS–Tween (PBS + 0.05% Tween 20) and the plates were incubated at 37° for 30 min between each step. Fetal bovine serum was used to block unreacted sites. Biotin-labeled goat anti-mouse IgG and horseradish peroxidase labeled streptavidin were used. The plates were then developed with a freshly made substrate solution (ATBS and H_2O_2). Plates were read after 10 to 30 min at 630 nm with a Dynatech MR 700. Controls were run simultaneously on the same plates.

Results

CarbE in rat and guinea pig small intestine. The CarbEs of the small intestine from both the rat and guinea pig were separated by chromatofocusing into three different isoenzymes. The small intestine preparation from the rat was separated into one main peak, pI 5.4, and a smaller one, pI 4.75. A third isoenzyme, pI <4.2, was detected after elution with 1 M NaCl solution. Similarly the small intestine preparation from the guinea pig was separated into two main peaks, pI 5.6 and 4.5, of CarbE activity and a small peak, pI <4.2, which could be detected after elution with 1 M NaCl solution.

Preparation and selection of MAb directed against rat lung CarbE (pI 5.8). The seven positive subclones, which were selected after an ELISA screening specific for CarbE (pI 5.8), were transferred to growth medium for large scale production. Only two of the cell cultures grew properly. The purified MAbs dissociated to only one heavy and one light chain in a SDS polyacrylamide gel, which supports the monoclonal nature of these antibodies.

ELISA in determination of immunological relationship. Non-competitive ELISA was used to assay for potential binding of two MAbs produced against CarbE (pI 5.8) toward the rat and guinea pig CarbE isoenzymes (Table 1). If binding of MAbs to the CarbE isoenzyme-coated microtest plates was less than 5% of control, the reaction was considered negative. For accurate results where cross-reactions were detected, it was essential to perform the ELISA under conditions where CarbE concentrations were proportional to each MAb. This was determined for each MAb and its reaction to purified rat lung CarbE (pI 5.8) before testing against other CarbEs. For each of the CarbE isoenzymes a sequence of dilutions was coated to microtest plates and tested against each MAb (see Fig. 1).

The results indicate the existence of common antigenic sites on rat CarbEs from lung (pI 5.8), liver (pI 6.2 and 6.0), plasma (pI 4.4) and small intestine (pI 5.4), and guinea pig CarbEs from plasma (pI 6.2) and small intestine (pI 5.6). Other CarbE isoenzymes reacted with only one of the MAbs and had therefore a weaker degree of similarity to the CarbE (pI 5.8). Table 1 also shows that MAb 1C1-1 reacted with two of the isoenzymes from the plasma and small intestine in the guinea pig, but the MAb 2H6-1 reacted only with the isoenzymes of highest pI. The results presented here indicate that 2H6-1 reacted better with the rat isoenzymes than with the guinea pig isoenzymes. The two MAbs did not bind to the CarbE isoenzymes purified from liver and lung in the guinea pig, except for one form (lung CarbE, pI 5.9).

Effect of MAbs on the catalytic activity of rat lung CarbE (pI 5.8). Each of the seven MAbs was tested for its ability to inhibit the hydrolysis of 4-nitrophenyl butyrate using rat

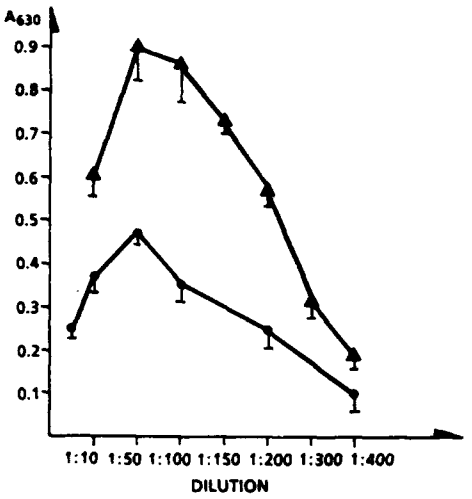

Fig. 1. ELISA for testing the linear range for different dilutions of CarbE (pI 6.0) from rat liver against two MAbs: 2H6-1 (▲) and 1C1-1 (●). Results are means ± SEM (N = 4).

lung CarbE (pI 5.8), but no inhibition was observed for these MAbs. These results indicate that the active site of the enzyme is not involved in the formation of the antigen–antibody complex.

Discussion

In rat liver four CarbE isoenzymes can be separated easily [7, 8]. These isoenzymes all behave as monomers with a molecular mass of about 60 kDa, except for CarbE (pI 6.0) which occurs as a stable trimer of about 180 kDa [7]. The CarbE (pI 6.0) reacted strongly with both MAbs (see Fig. 1 and Table 1). This isoenzyme also resembles rat lung CarbE (pI 5.8) in number of subunits, molecular mass and the N-terminus [6]. The other rat liver CarbE isoenzymes have at least one epitope in common, illustrated by the relatively high binding of the isoenzymes to the MAb 2H6-1.

Two different reports on the purification of CarbE from rat plasma have been published. Choudhury [9] reported a molecular mass of 43 kDa, but Hashinotsume *et al.* [10] reported a highly purified CarbE with a molecular mass of 84 kDa and pI 4.4. The discrepancy between the two molecular masses can be explained by the possibility that the enzyme was dissociated into subunits of 43 kDa, which were also active. Our immunological results show that there have to be two different CarbE isoenzymes with separate protein structures, as only CarbE (pI 4.4) was bound to the MAbs. The subunit masses of two of the guinea pig plasma CarbEs (pI 6.2 and pI <4.0) have been determined to be 58 and 80 kDa [11]. Both MAbs reacted strongly with guinea pig plasma CarbE (pI 6.2), but there were no significant reaction of the low pI form CarbE (pI <4.0) with the MAbs.

De Jong *et al.* [12] have shown that antiserum raised against a rat liver CarbE hydrolysing monoglyceride inhibits one of the three CarbEs from rat small intestine completely. These two CarbE isoenzymes, both showing monoacylglycerol hydrolase activity, were therefore suggested to be at least partially similar. Mentlein *et al.* [1] have demonstrated that an antiserum to rat liver CarbE (pI 6.0) gave a precipitation with rat serum. In agreement, our immunological results also indicate a relationship between CarbE from rat liver and the high pI forms of CarbE in the small intestine and plasma. However, the lack of response of the low pI forms indicates that two distinct categories of CarbE isoenzymes are present in both the rat and guinea pig, as shown earlier in the mouse [13].

Böcking and von Deimling [14] have suggested that one or two different CarbE isoenzymes from mouse jejenum enter the blood stream with the chylomicrons. Sterri [15] has proposed that intestinal CarbE may be a possible source of the enzyme in rat plasma, due to the oxime-induced reactivatibility and the low pI for enzyme in both tissues. Our immunological results could support such a proposal.

In summary, the immunological study showed a strong relationship between the rat lung CarbE (pI 5.8) and the rat liver CarbE (pI 6.0), but very little similarity of these CarbEs to the guinea pig liver and lung CarbE isoenzymes. The MAbs were strongly bound to the high pI forms of the CarbE isoenzymes in the plasma and small intestine from both the rat and guinea pig, but not with the low pI forms. These results indicate that two immunochemically distinct categories of CarbE isoenzymes are present in the plasma and small intestine.

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Paper IV

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Heterogeneity of carboxylesterases in rat liver cells

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Abstract—Rat liver cells were separated into parenchymal cells (PC), Kupffer cells (KC) and endothelial cells (EC). The distribution of carboxylesterases (EC 3.1.1.1) between these cell types was investigated by PAGE and chromatogenic substrate staining, and compared with the results for total liver preparation and individual isoenzymes isolated by chromatofocusing. All of the liver carboxylesterase isoenzymes could be detected in the PC, whereas in both KC and EC only those with isoelectric point (pI) 6.4/6.2 could be detected. Use of carboxylesterase inhibitors like bis-(4-nitrophenyl)phosphate and paraoxon, and organophosphorus compound hydrolase inhibitors like 4-hydroxymercuribenzoate and EDTA confirmed that these esterases were of the carboxylesterase type.

In rat liver, carboxylesterase (EC 3.1.1.1) activity is localized within both the endoplasmatic reticulum and the cytosol [1], but the microsomal carboxylesterase isoenzymes have been characterized most extensively [2]. Six carboxylesterases have been purified from rat liver microsomes by isoelectric focusing or by chromatofocusing and named by their isoelectric points (pI 6.4, 6.2, 6.0, 5.6, 5.2 and 5.0) [3]. Four of the esterases have different primary structures and differ in their specificity for ester and amide substrates [4]. Liver carboxylesterases are strongly involved in the metabolism of xenobiotics, and natural substrates have also been reported [5].

It is known that 60% of the liver cells are parenchymal cells (PC*), 22% endothelial cells (EC) and 10% Kupffer cells (KC) [6]. By electron microscopic autoradiography of liver slices labeled with [^{3}H]diisopropyl phosphorofluridate it was demonstrated that the bulk of organophosphate-sensitive esterase sites were localized in the PC, the Kupffer cells were not significantly different from the background [7].

The problem concerning the individual localization of enzymes in different types of cells is often neglected, and the present study was undertaken to examine whether each type of liver cell has its own carboxylesterase pattern.

Materials and Methods

Chemicals. Acrylamide, *N,N'*-methylene-bis-acrylamide, ammonium persulphate and TEMED were from Bio-Rad (Richmond, CA, U.S.A.). 4-Nitrophenyl butyrate, α-naphthyl acetate, Fast Blue B Salt, bis-(4-nitrophenyl)-phosphate and 4-hydroxymercuribenzoate were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Paraoxon (diethyl 4-nitrophenyl phosphate) was from Koch-Light Laboratories (Colnbrook, U.K.). Nycodenz was obtained from Hafslund Nycomed A/S (Oslo, Norway). All other chemicals were of analytical-grade quality.

Animals. Male Wistar rats (200–250 g) were used for all experiments. The animals were purchased from the National Institute of Public Health, Oslo, Norway. The animals were given a standard laboratory diet and water *ad lib.*

Preparation of PC, KC and EC. Experimental animals were anesthetized with pentobarbital, and total liver cell suspension was obtained by enzymatic perfusion of the rat liver [8] following a modified two-step procedure [9]. The liver cell suspension was filtered through nylon gauze and sedimented by centrifugation (2 min, 16 *g*). The pellet containing PC was twice resuspended in incubation buffer [10] and centrifuged.

Non-parenchymal liver cells were sedimented from the supernatant by centrifugation (4 min at 310 *g*) according to Magnusson *et al.* [11]. The cells were resuspended in 50 mL of incubation buffer containing 1% BSA and centrifuged (2 min, 16 *g*), and the resulting supernatant was recentrifuged (4 min, 310 *g*). Any PC remaining in the pellet were quantitatively removed by centrifugal elutriation at 1500 rpm. The non-parenchymal cells were separated further by centrifugal elutriation at 2500 rpm, and the EC were collected in 100 mL at a flow rate of 22 mL/min. The sediment (containing the KC) was resuspended in incubation buffer containing 20% (w/w) Nycodenz and carefully overlaid with incubation buffer containing 1% BSA. The KC were collected from the interface after centrifugation (15 min, 1450 *g*), washed and resuspended in incubation buffer containing 1% BSA. The EC were further purified by resuspending the cells in incubation buffer containing Nycodenz as described for the KC preparation.

Cell viability was assessed by the Trypan blue exclusion test, and the purity of PC, KC and EC was found to be at least 99% [12]. The PC, KC and EC were frozen before they were dissolved in 20 mM Tris–glycine, pH 8.5, containing 2.0 M glycerol, and applied to the gels.

Preparation of rat liver fraction for chromatofocusing. Liver from one to two animals was rinsed with ice-cold 50 mM Tris, pH 7.5, containing 0.1 M NaCl and homogenized in 4 vol. 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 high-speed supernatant was fractionated by ammonium sulphate precipitation. The 35–70% saturated ammonium sulphate fraction was desalted by eluting through Sephadex G-25M (Pharmacia PD-10 column) and applied to a chromatofocusing column as described previously [13].

PAGE. The gels were run and stained by the method of Øien and Stenersen [14]. The PAGE was run with a Mini Protean II cell from Bio-Rad. Gel slabs 7 × 8 cm^2, 1.00 mm thick were used with 7.5% acrylamide and 0.25% *N,N'*-methylene-bis-acrylamide (stacking gel: 3.0% acrylamide and 0.10% *N,N'*-methylene-bis-acrylamide) in 0.375 M Tris–HCl buffer, pH 8.8 (stacking gel-buffer: 0.125 M Tris–HCl, pH 6.8). Tris–glycine (5 mM, pH 8.5) was used as electrode buffer. Samples of 10–20 μL were applied after addition of Bromophenol blue (0.01%) as marker. A Pharmacia LKB model power supply was used (190 V for approximately 1.5 hr).

The staining solution contained 50 mM Tris–HCl buffer, pH 8.0 and 10 mg of α-naphthyl acetate in 1 mL acetone per 100 mL buffer, and 50 mg Fast Blue B salt per 100 mL

* Abbreviations: PC, parenchymal cells; KC, Kuppfer cells; EC, endothelial cells; BSA, bovine serum albumin.

buffer was added as a powder. The bands developed for 20 min at room temperature.

Inhibitors like paraoxon (1.0×10^{-4} M), bis-(4-nitrophenyl)phosphate (2.0×10^{-4} M), 4-hydroxymercuribenzoate (1.0×10^{-3} M) and EDTA (1.0×10^{-3} M) were used in parallel runs to non-inhibited gels. These inhibitors were added 1 hr before addition of substrate and Fast Blue B Salt.

Other analytical methods. Carboxylesterase activity was measured spectrophotometrically with 4-nitrophenyl butyrate as substrate [15].

Protein was determined as described by Lowry *et al.* [16], with BSA as standard.

Results

Total carboxylesterase activity in different types of rat liver cell is given in Fig. 1. These results illustrate that the main carboxylesterase activity is localized in the PC, but activity is also present in KC and EC. Therefore, it was of interest to examine the distribution of the different isoenzymes of carboxylesterase in each of the liver cell types.

Figure 2 illustrates a non-denaturing polyacrylamide gel stained with α-naphthyl acetate and Fast Blue B salt. The purified fraction of PC migrated as a cascade of distinct bands (lane 2). The slowest migrating band corresponded to the isoenzyme pI 6.0, which occurs as a trimer in a non-denaturing gel (lane 7). The next band has been described as being the monomeric form of the isoenzyme pI 6.0 [17]. The isoenzymes with pI 6.4/6.2 migrated as two bands, whereas the isoenzyme pI 5.6 appeared heterogeneous. A comparison of the PC fraction (lane 2) with the homogenate fraction (lane 1) shows that the bands were nearly identical, with differences in the intensity of staining only. This illustrates that the PC contain all of the main forms of carboxylesterase which can be isolated from rat liver. The fractions of KC (lane 3) and EC (lane 4) migrated identically, but with only two main bands. These two protein bands were similar to the pI 6.4/6.2 (lane 6). In some instances we could see traces of the band corresponding to isoenzyme pI 6.0. The fastest migrating band (a weak band at the bottom of the gel in lanes 3 and 4) did not disappear when the gel was treated with the inhibitors paraoxon and bis-(4-nitrophenyl)phosphate, but did when treated with 4-hydroxymercuribenzoate and EDTA, which confirms that this protein was not of the carboxylesterase type.

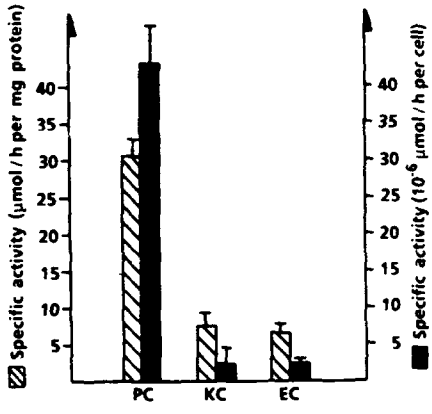

Fig. 1. Distribution of carboxylesterase activity in rat liver cells. Results are means ± SEM (N = 4).

Discussion

Isoenzyme pI 6.0, the predominant esterase in rat liver parenchymal cells, has the highest activity towards short aliphatic esters and also butanilicaine [3]. This isoenzyme is probably involved in the detoxication of xenobiotics, but also in the metabolism of natural substrates. In the EC and KC this isoenzyme appears to be of minimal importance.

In sharp contrast to the heterogeneity of carboxylesterases in PC, the KC and EC contain mainly the isoenzyme pI 6.4/6.2. These two closely related isoenzymes hydrolyse retinyl palmitate, palmitoyl-CoA and monoacylglycerols. The two forms, coded by one gene locus (ES-4B), hardly differ in their catalytic properties [18]. They possess an identical amino acid sequence, but possibly these two forms differ in their glycosylation [2]. Robbi and Beaufay [19] have identified two active enzyme forms of pI 6.4 with slightly different polypeptide chain lengths, but immunoblots revealed a single form with $M_r \approx 62{,}000$ and they explained the heterogeneity as a result of proteolysis, without inactivation of the enzyme. the PAGE results for the isolated pI 6.4/6.2 fraction show two separate bands (lane 6). Surprisingly, the pI 6.4 fraction changed from migrating like the slowest band to migrating like the fastest band of the pI 6.4/6.2 fraction (lane 6), when the solution was kept for some months at 4° (shown in lane 5). This observation

Fig. 2. Esterase banding patterns following PAGE of rat liver. The gel was stained with α-naphthyl acetate. Lane 1, rat liver extract; lane 2, PC; lane 3, KC; lane 4, EC; lane 5, carboxylesterase pI 6.4; lane 6, carboxylesterase pI 6.4/6.2; lane 7, carboxylesterase pI 6.0.

agrees with the findings of Robbi and Beaufay [19] that a peptide chain is cleaved from the rest of the pI 6.4 isoenzyme. A comparison of the isolated pI 6.4/6.2 isoenzymes with correlating bands for PC and liver homogenate illustrate differences in electrophoretic mobility. The mismatching might well be generated by differences in proteolysis of the authentic isoenzymes.

Only the PC show the three bands corresponding to isoenzyme pI 5.6 (lane 2). The differences in relative mobilities are due to the heterogeneity at the N-terminal end [3]. The isoenzymes pI 5.2/5.0 (not shown in Fig. 2) were detected in both PC and homogenate when the staining time was considerably extended (from 40 to 60 min).

The metabolic function of the PC depends on their specific position in the liver, and each individual cell probably has the potential for all hepatic functions, including the detoxication reactions. This illustrates the versatile functions among the PC compared with the more specialized functions of the KC and EC. In relation to these properties a heterogeneous pattern of the carboxylesterases in PC would be expected.

Both KC and EC are able to endocytose a large variety of different particles and molecules, and they contain high specific activities of lysosomal enzymes [20]. KC and EC are in direct contact with the bloodstream and they constitute a coordinated defence system that protects PC against injury [21]. The carboxylesterase pI 6.2/6.4, also termed monoacylglycerol lipase (EC 3.1.1.23) [22], in both EC and KC may serve to protect the liver by hydrolysis of the long chain monoacylglycerols in the degradation of lipopolysaccharides.

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