The identification and characterization of two separate carboxylesterases in guinea-pig serum

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The esterase activity of guinea-pig serum was investigated. A 3-fold purification was achieved by removing the serum albumin by Blue Sepharose CL-6B affinity chromatography. The partially purified enzyme preparation had carboxylesterase and cholinesterase activities of 1.0 and 0.22 μmol of substrate/min per mg of protein respectively. The esterases were labelled with [^3H]di-isopropyl phosphorofluoridate (DiPF) and separated electrophoretically on sodium dodecyl sulphate/polyacrylamide gels. Two main labelled bands were detected: band I had M_r 80 000 and bound 18–19 pmol of [^3H]DiPF/mg of protein, and band II had M_r 58 000 and bound 7 pmol of [^3H]DiPF/mg of protein. Bis-*p*-nitrophenyl phosphate (a selective inhibitor of carboxylesterase) inhibited most of the labelling of bands I and II. The residual labelling (8%) of band I but not band II (4%) was removed by preincubation of partially purified enzyme preparation with neostigmine (a selective inhibitor of cholinesterase). Paraaxon totally prevented the [^3H]DiPF labelling of the partially purified enzyme preparation. Isoelectrofocusing of [^3H]DiPF-labelled and uninhibited partially purified enzyme preparation revealed that there were at least two separate carboxylesterases, which had pI 3.9 and pI 6.2, a cholinesterase enzyme (pI 4.3) and an unidentified protein that reacts with [^3H]DiPF and has a pI 5.0. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of these enzymes showed that the carboxylesterase enzymes at pI 3.9 and pI 6.2 corresponded to the 80 000- M_r subunit (band I) and 58 000- M_r subunit (band II). The cholinesterase enzyme was also composed of 80 000- M_r subunits (i.e. the residual labelling in band I after bis-*p*-nitrophenyl phosphate treatment). The unidentified protein at pI 5.0 corresponded to the residual labelling in band II (M_r 58 000), which was insensitive to neostigmine and bis-*p*-nitrophenyl phosphate. These studies show that the carboxylesterase activity of guinea-pig serum is the result of at least two separate and distinct enzymes.

Carboxylesterases (EC 3.1.1.1) are a group of enzymes that hydrolyse a wide range of carboxylic esters and are important in the hydrolytic transformation of many toxic pesticides, insecticides and

drugs. Studies on carboxylesterases are difficult owing to the lack of pure homogeneous enzyme preparations, multiplicity of enzyme forms and the wide and often overlapping substrate specificity (for reviews see Junge & Krisch, 1975; Ahmad & Forgash, 1976; Heymann, 1980). These factors make even the enzyme nomenclature a difficult problem, although the original A- and B-esterase classification proposed by Aldridge (1953) is still appropriate. [B-esterases are inhibited by paraaxon (diethyl 4-nitrophenyl phosphate), whereas A-esterases are not and hydrolyse organophosphorus esters]. Carboxylesterases are found in many tissues, including the liver (usually the most abundant source), serum and kidneys (Junge & Krisch, 1975).

Abbreviations used: DiPF, di-isopropyl phosphorofluoridate; SDS, sodium dodecyl sulphate.

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Most of the B-esterase activity of the liver is located in the microsomal fraction, and purification procedures require an initial solubilization step. A large number of liver carboxylesterase preparations have been described (Junge & Krisch, 1975; Heymann, 1980), and even allowing for differences in procedures and nomenclature it is clear that there are many forms of liver carboxylesterase. A much simpler situation exists in serum, as Aldridge (1953) showed that certain species (notably the rat and guinea pig) have very high B-esterase activity and only a small amount of A-esterase. Unfortunately, there are few data on these soluble esterases, and to date only the rat enzyme has been purified, by Choudhury (1974) and Hashinotsume *et al.* (1978). The latter showed that the enzyme had M_r 84000, whereas Choudhury (1974) reported that his preparation had M_r 43000. These results suggest that there are at least two forms of B-esterase in rat serum. In our studies we have used the guinea pig, and the experiments reported in the present paper describe the characterization of the B-esterases in serum. To this end [1,3- ^3H]DiPF has been used as a radioactive label for B-esterases that have been separated and identified by a combination of SDS/polyacrylamide-gel electrophoresis and isoelectrofocusing. These techniques, in conjunction with the use of other selective esterase inhibitors, demonstrate that guinea-pig serum has at least two carboxylesterase enzymes with quite different pI values and subunit molecular masses.

Materials and methods

Chemicals

All reagents for SDS/polyacrylamide-gel electrophoresis were obtained from BDH Chemicals, Poole, Dorset, U.K., and were specially purified for electrophoresis. Blue Sepharose CL-6B, Sephadex G-25 and Low Molecular Weight (LMW) Electrophoresis Calibration Kits were obtained from Pharmacia, Hounslow, Middx., U.K. [1,3- ^3H]DiPF (specific radioactivity 222 GBq/mmol) was obtained from Amersham International, Amersham, Bucks., U.K., as a solution in propylene glycol. The stock solution (0.2 ml) was then incubated at 37°C for 20 h in 0.85 ml of 0.2 mM-EDTA/50 mM-Tris/citrate buffer, pH 6.0, to remove impurities (Clothier & Johnson, 1979). The resulting solution was stored at -20°C. Horse serum cholinesterase (type IV), pig liver esterase (type II), *S*-butyrylthiocholine iodide and neostigmine were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. Bis-*p*-nitrophenyl phosphate was obtained from the Aldrich Chemical Co., Gillingham, Dorset, U.K. Phenyl butyrate was synthesized as described by Aldridge

(1953). All other chemicals were of AnalaR grade from BDH Chemicals.

Preparation of guinea-pig serum

Blood was removed by heart puncture from female Dunkin Hartley guinea pigs (300–500 g; OLAC, Bicester, Oxon, U.K.) that had been killed by CO₂ gassing. The blood was then transferred to centrifuge tubes and allowed to clot at 37°C for 1 h before being centrifuged at 3000 rev./min for 10 min in an MSE bench centrifuge. The supernatant (serum) was then carefully removed by aspiration and stored at -20°C.

Preparation of partially purified enzyme

The protein concentration of the serum was 50–60 mg/ml, a large proportion of which is serum albumin (approx. 70%). A procedure was therefore developed to remove the albumin by Blue Sepharose CL-6B affinity chromatography. The packing material for this column consists of the dye Cibacron Blue F3-GA conjugated to Sepharose. As shown by Travis *et al.* (1976), this has a very high affinity for albumin. In the present study a 30 cm × 2.5 cm column of Blue Sepharose CL-6B equilibrated in buffer A (100 mM-ammonium formate, pH 8.0) at 5°C and eluted at a flow rate of 10 ml/h was used to remove albumin from guinea-pig serum. Preliminary runs showed that the column would bind 450 mg of bovine serum albumin (in buffer A), which could be then eluted with 1 M-ammonium formate (buffer B) and good recovery (>95%). When guinea-pig serum was processed in this way, 90% and 95% of the carboxylesterase and cholinesterase activity respectively were eluted at between 90 ml and 150 ml of buffer A, and this fraction also contained 30% of the applied protein. The remaining protein (albumin) was eluted with buffer B, and no enzymic activity was detected in any of these fractions. Those fractions containing carboxylesterase and cholinesterase activity were pooled (referred to below as 'partially purified enzyme preparation'), freeze-dried and stored at -20°C. All subsequent experiments used the partially purified enzyme preparation as a starting material.

Measurement of enzyme activity

Carboxylesterase activity. Carboxylesterase activity was measured at 25°C in 100 mM-sodium phosphate buffer, pH 7.4, with phenyl butyrate (1 mM final concentration) as the substrate (Verschoyle *et al.*, 1982). Enzyme was incubated in buffer (2.7 ml) before initiation of the reaction with 0.3 ml of substrate (in 5% ethanol) and detecting the liberated phenol at 270 nm (ϵ 1510 M⁻¹·cm⁻¹ in a Pye-Unicam SP.8100 recording spectrophotometer (Pye-Unicam, Cambridge, U.K.).

Cholinesterase activity. Cholinesterase activity was determined at 25°C in 100mM-sodium phosphate buffer, pH7.4, by the spectrophotometric method of Ellman *et al.* (1961), with butyrylthiocholine iodide as substrate.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

SDS/polyacrylamide-gel electrophoresis

Vertical slab-gel electrophoresis was performed in the presence of 0.1% (w/v) SDS as described by Douglas & Butow (1976) with the discontinuous buffer system of Laemmli (1970) in an LKB 2001 vertical electrophoresis apparatus (LKB Instruments, Croydon, Surrey, U.K.). The stacking gel was 5% acrylamide and the resolving gel a 7½–15% exponential gradient of acrylamide. The gels were run at 30mA constant current at 5°C until the Bromophenol Blue tracking dye had run approx. 11cm into the resolving gel (about 5h). After electrophoresis the dye front was marked, and the gel was fixed for 30min in 50% (w/v) trichloroacetic acid, stained for 30min with 0.1% PAGE Blue 83 dye in 40% (w/v) trichloroacetic acid and destained overnight in 7.5% (v/v) acetic acid. After the destaining, the gels were photographed and scanned at 575nm in a Chromoscan (Joyce Loebel, Gateshead, Tyne and Wear, U.K.).

Enzyme samples (2mg/ml) for SDS/polyacrylamide-gel electrophoresis were usually dissolved in an equal volume of 50mM-Tris/HCl buffer, pH6.8, containing 2% (w/v) SDS, 2mM-EDTA, 1% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol and 0.01% (w/v) Bromophenol Blue (sample preparation buffer). Before being loaded on the gel (20µl per well), the samples were heated at 60°C for 30min. Protein standards (Pharmacia LMW calibration kit) were reconstituted in 100µl of the sample preparation buffer and heated at 60°C for 30min before being loaded (5µl) on the gel.

Radioactively labelled gels were assayed for ³H by cutting out the appropriate destained gel track (1cm strip) and freezing in solid-CO₂ powder before slicing it into 1mm sections with a Mickle gel slicer (Mickle Engineering Co., Gomshall, Surrey, U.K.). Each slice was digested with 0.25ml of H₂O₂ (30%, w/v) at 60°C for 12h in a tightly capped scintillation vial. The vials were then cooled to 5°C (to condense any ³H₂O vapour) before the addition of 0.75ml of water and 10ml of Instagel scintillation fluid (Packard Instruments, Caversham, Berks., U.K.).

Isoelectrofocusing on granulated gel

Granulated gels were prepared and electrophoresed as described in the *LKB Application Note 198* by using the method of Radola (1973, 1974). The

initial gel slurry contained 2% (w/v) Ampholines (LKB; 1vol. of pH2.5–4 Ampholine plus 3vol. of pH3.5–10 Ampholine), 10% (v/v) glycerol and 4% (w/v) Ultradex gel (LKB) in deionized water. The gel was dried to the correct weight and prefocused at 8W (constant power) for 30min at 6°C. The enzyme sample was applied as a band near the cathode, and the gel was electrophoresed at 8W (constant power) for 16h at 6°C. After the electrofocusing, the gel was sectioned into 30 slices, which were eluted with 10% (v/v) glycerol (2ml per slice). The pH of the eluate was then measured before adjustment of the pH to 7.4 with 0.2ml of 0.1M-sodium phosphate buffer. Appropriate samples were then taken for measurement of enzyme activity and radioactivity (see the Results section). For SDS/polyacrylamide-gel electrophoresis, appropriate samples were concentrated in Minicon Multipurpose Microconcentrators (*M_r* cut-off 15000; Amicon, High Wycombe, Bucks., U.K.) to 0.5ml, resuspended to 5ml with water and reconstituted to 0.5ml. This was then rediluted to 5ml with 50mM-Tris/HCl buffer, pH6.8, containing 2mM-EDTA and 0.1% (w/v) SDS, and finally concentrated to approx. 100µl. This final concentrated sample was free of Ampholines and was prepared for SDS/polyacrylamide-gel electrophoresis by adding 100µl of SDS sample preparation buffer and heating at 60°C for 30min.

[³H]DiPF labelling studies

Esterases of serum and the partially purified enzyme preparation were radioactively labelled with [³H]DiPF by incubation of the sample (2mg/ml final protein concentration) in 10mM-ammonium formate buffer, pH8.0, for 5min at 37°C. In the SDS/polyacrylamide-gel-electrophoresis experiments, the labelling reaction was terminated by the addition of an equal volume of SDS sample preparation buffer and heating at 60°C for 30min. The labelled preparation was then applied directly to the gels without prior removal of unchanged [³H]DiPF. In some experiments (isoelectrofocusing) the labelling was terminated by rapidly cooling the mixture to 0°C and then removing the unchanged [³H]DiPF by desalting on a small Sephadex G-25 column (8cm×0.6cm) equilibrated in 100mM-ammonium formate buffer, pH8.0. Under these conditions the esterase activity of the labelled partially purified enzyme preparation (after the Sephadex G-25 treatment) was more than 95% inhibited. Furthermore, mixing the labelled partially purified enzyme preparation with the uninhibited preparation did not produce any inhibition, demonstrating that all free [³H]DiPF had been removed by the Sephadex G-25 treatment.

Initially, labelling was performed with a [³H]DiPF

concentration of $71\mu\text{M}$ and incubation time of 60 min. However, subsequent experiments showed that maximum labelling was achieved within 5 min and that the $[^3\text{H}]\text{DiPF}$ concentration could be decreased to $1\mu\text{M}$. For inhibition studies, the enzyme preparation was preincubated with the appropriate inhibitor for 30 min at 37°C before the addition of $[^3\text{H}]\text{DiPF}$.

Measurement of radioactivity

Radioactivities of samples were usually counted for 10 min in a Beckman LS6800 series scintillation counter (Beckman, High Wycombe, Bucks., U.K.), equipped with a chemiluminescence monitor. In some cases, radioactivities of samples with low radioactivity were re-counted for 100 min to ensure accuracy. Any chemiluminescent samples (usually in digested gel slices, due to residual H_2O_2) had their radioactivities re-counted after a suitable period of storage in the dark.

Results

Guinea-pig serum had routine carboxylesterase and cholinesterase specific activities of approx. 0.4 and $0.08\mu\text{mol}$ of substrate/min per mg of protein respectively. Removal of serum albumin by affinity chromatography resulted in a partially purified enzyme preparation with higher specific activities. In a typical column run the carboxylesterase activity increased nearly 3-fold from 0.38 (sera) to $1.0\mu\text{mol}$ /min per mg of protein (partially purified preparation). A similar increase was seen with the cholinesterase activity. A comparison of the partially purified enzyme preparation with guinea-pig serum by SDS/polyacrylamide-gel electrophoresis showed that the Blue Sepharose CL-6B chromatography removed all of the albumin and thus provided an easy method for the removal of 70% of the non-active protein from the serum.

$[^3\text{H}]\text{DiPF}$ is a covalent inhibitor of all B-esterases and can therefore be used to radiolabel the protein subunits, which can then be analysed by SDS/polyacrylamide-gel electrophoresis. This approach has been used in the present study, and Fig. 1 shows an SDS/polyacrylamide-gel electrophoretogram of $[^3\text{H}]\text{DiPF}$ -labelled partially purified enzyme preparation. Two labelled bands were detected, and protein standards were used to determine the M_r values of the proteins. Band I had M_r approx. 80000 and a site concentration of 18–19 pmol of $[^3\text{H}]\text{DiPF}$ /mg of protein. Band II was smaller, with M_r 58000, and had a site concentration of about 7 pmol of $[^3\text{H}]\text{DiPF}$ /mg of protein. The M_r determinations were performed on gradient gels, but similar values were obtained when fixed-concen-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of $[^3\text{H}]\text{-DiPF}$ -labelled partially purified enzyme preparation. The partially purified enzyme preparation (2 mg of protein/ml) was labelled with $[^3\text{H}]\text{DiPF}$ ($71\mu\text{M}$ for 30 min), and the labelled protein subunits (P.P.E.) were separated by SDS/polyacrylamide-gel electrophoresis in a 7½–15% exponential acrylamide gradient gel as described in the Materials and methods section. The stained gel slice (20 μg of protein) and densitometer scan (A_{575}) are shown above. M_r standards were used to calibrate the gel: 1, phosphorylase b, M_r 94 000; 2, bovine serum albumin, M_r 67 000; 3, ovalbumin, M_r 43 000; 4, carbonic anhydrase, M_r 30 000; 5, trypsin inhibitor, M_r 20 100; 6, α -lactalbumin, M_r 14 400. The histogram shows the ^3H radioactivity content of each 1 mm slice. For clarity the two labelled peaks are marked I (M_r 80 000) and II (M_r 55 000–60 000).

tration gels were used (i.e. 7½% and 10% acrylamide gels). These results suggested that one labelled band corresponded to the carboxylesterase activity and the other to the cholinesterase activity. These results, however, did not differentiate between the various types of esterase, as they were all tritiated by this procedure. This problem was overcome by the use of selective irreversible inhibitors to mask the appropriate esterase active site (see, e.g., Scheiner & Quigley, 1982; Williams & Johnson, 1981). Thus experiments were performed in which the partially purified enzyme preparation was preincubated with neostigmine, a selective inhibitor of cholinesterase (Koelle, 1970). Table 1(a) shows that various concentrations of neostigmine totally inhibited the cholinesterase enzyme without affecting the car-

Table 1. *Effects of neostigmine, bis-p-nitrophenyl phosphate and paraoxon on [³H]DiPF labelling of the partially purified enzyme preparation, horse serum cholinesterase and pig liver esterase*
For experimental details see the text.

Concn. of inhibitor (mM)	Inhibition of labelling (%)				
	Partially purified enzyme preparation		Pig liver esterase (M_r 63 000)	Horse serum cholinesterase	
	Band I (M_r 80 000)	Band II (M_r 58 000)		Band A (M_r 120 000)	Band B (M_r 83 000)
Neostigmine					
0.1	23 (1)	20 (1)	—	—	—
1	17 (1)	9 (1)	—	—	—
2	—	—	22	100	100
10	3(2)	14 (2)	—	100	97
Bis- <i>p</i> -nitrophenyl phosphate					
1	99 (2)	94 (2)	100	0	0
Paraoxon					
0.2	100 (1)	100 (1)	—	—	—

boxylesterase activity. However, there was little effect on the [³H]DiPF-labelled bands of the partially purified enzyme preparation and the pig liver esterase preparation (Sigma). In contrast, the horse serum cholinesterase (Sigma) was totally inhibited by neostigmine (results not shown), and furthermore this was correlated with a complete inhibition of [³H]DiPF labelling. In Table 1(b) the effect of bis-*p*-nitrophenyl phosphate is shown, this compound being a selective inhibitor of carboxylesterase (Heymann & Krisch, 1967; Junge & Krisch, 1975). The [³H]DiPF-labelled pig liver esterase peak was completely inhibited, as also were both labelled bands in the partially purified enzyme preparation. Conversely, the horse serum cholinesterase enzyme was not inhibited by bis-*p*-nitrophenyl phosphate (results not shown), which also did not have any effect on the radiolabelling. Table 1(c) shows that paraoxon, which is an inhibitor of all B-esterases, totally abolished the [³H]DiPF labelling of the partially purified enzyme preparation, and thus both labelled peaks were due to B-esterases.

These results demonstrated that the major proportion of the radioactivity associated with band I and band II of the labelled partially purified enzyme preparation was due to carboxylesterase and not the cholinesterase activity, and that there were possibly two separate carboxylesterase enzymes. However, these experiments did not give information on the cholinesterase enzyme, which did not appear to be labelled by [³H]DiPF. A possible explanation for this result is that the enzyme has a high turnover and therefore the concentration of cholinesterase in the partially purified preparation is very low. The radioactive labelling would therefore be too low to detect on the SDS/polyacrylamide-gel electro-

phoretograms. However, when the amount of protein applied to the SDS/polyacrylamide gels was increased nearly 10-fold, the gel, although overloaded, still revealed bands I and II [compare Fig. 2(a) with Fig. 1] and also revealed another small band at M_r approx. 65 000. Band I bound 19.07 pmol of [³H]DiPF/mg of protein, and band II had 5.65 pmol of [³H]DiPF/mg of protein. Pretreatment with bis-*p*-nitrophenyl phosphate (Fig. 2b) decreased the labelling of band I and band II by 92% and 96% respectively to 1.48 and 0.22 pmol of [³H]DiPF/mg of protein. The remaining radioactivity bound to band I after bis-*p*-nitrophenyl phosphate was completely abolished by neostigmine (Fig. 2c). The residual activity in band II, however, was not affected by neostigmine. These results demonstrated that the 80 000 M_r labelled band was a mixture of two catalytic activities. Thus 92% of the radioactive label is due to [³H]DiPF binding to a carboxylesterase enzyme; the remaining [³H]DiPF is associated with the cholinesterase activity. In the case of the 58 000 M_r band a similar situation exists with 96% of the [³H]DiPF bound to a carboxylesterase enzyme. The residual activity is neither neostigmine- nor bis-*p*-nitrophenyl phosphate-sensitive, and is therefore probably not a cholinesterase or carboxylesterase enzyme even though it reacts with [³H]DiPF.

The SDS/polyacrylamide-gel-electrophoresis results showed that there were at least two carboxylesterases in guinea-pig serum, and this was investigated further by isoelectrofocusing in granulated gels. In the experiment described in Fig. 3 a mixture of [³H]DiPF-labelled and active partially purified enzyme preparations was separated by isoelectrofocusing. This procedure allowed the

Fig. 2. Effects of bis-*p*-nitrophenyl phosphate and neostigmine on [^3H]DiPF labelling of the partially purified enzyme preparation

In this experiment three separate incubations were carried out. (a) Partially purified enzyme preparation (4 mg of protein/ml) was incubated at 37°C for 30 min before the addition of [^3H]DiPF (1 μM) for 5 min to label the esterases; (b) as (a) except that the 30 min preincubation step was performed with the addition of bis-*p*-nitrophenyl phosphate (final concn. 1 mM); (c) as (a) except that the preincubation step was performed in the presence of 1 mM-neostigmine and 1 mM-bis-*p*-nitrophenyl phosphate. All three samples were then analysed by SDS/polyacrylamide-gel electrophoresis as indicated in the Fig. 1 legend except that 185 μg of protein per sample well was used.

simultaneous analysis of both enzyme activity and [^3H]DiPF labelling. As shown in Fig. 3, two asymmetrical peaks of carboxylesterase activity were focused at pI 3.9 (a) and pI 6.2 (d). The cholinesterase activity separated as a single broad peak at pI 4.3 (b), and this coincided with a shoulder on the carboxylesterase (a) peak. The spread of this peak suggests that the cholinesterase activity may be due to more than one enzyme, and in this context the shoulder on the carboxylesterase peak d may also be indicative of heterogeneity. The recovery of enzymic activity in this experiment was in the range 90–100% for both enzymes. The ^3H radioactivity profile correlated very closely with the carboxylesterase activity, except for a small peak at pI 5.0 (c), which

Fig. 3. Isoelectrofocusing of the partially purified enzyme preparation partially labelled with [^3H]DiPF

In this experiment both active and radiolabelled enzyme were co-isoelectrofocused, on a granulated gel. For this purpose a portion of the partially purified enzyme preparation (1.7 mg of protein) was labelled with [^3H]DiPF and the unchanged inhibitor was removed by Sephadex G-25 chromatography (see the Materials and methods section). The labelled preparation was then mixed with 17.1 mg of protein of active (non-inhibited) partially purified enzyme preparation, and the mixture was isoelectrofocused as described in the Materials and methods section. The eluted fractions were analysed for ^3H radioactivity (○), carboxylesterase activity (●), cholinesterase activity (▲) and pH (△). The indicated fractions were pooled to give four preparations (a, b, c and d), which were pooled and stored at -20°C before SDS/polyacrylamide-gel electrophoresis (see Fig. 4).

did not have carboxylesterase or cholinesterase activity. The recovery of radioactivity in this and other labelling experiments was approx. 60%. The non-recovered ^3H radioactivity migrated to the anode and was not associated with any enzymic activity. The four peaks (a, b, c and d) were then analysed by SDS/polyacrylamide-gel electrophoresis after the Ampholines had first been removed by repeated concentration and dilution in Minicon concentrators (see the Materials and methods section). Radioactivity recovery in this procedure was approx. 68%. The gel profiles shown in Fig. 4 indicated that the carboxylesterase enzymes, which focused at pI 3.9 (i.e. peak a) and 6.2 (i.e. peak d), had subunits of M_r 80 000 and M_r 58 000 respectively. The interpretation of peak b (pI 4.3), which contains the cholinesterase enzyme, is more complicated, owing to the overlapping enzyme activities (see Fig. 3). However, this problem was resolved in other isoelectrofocusing experiments, in which the partially purified enzyme preparation was inhibited with bis-*p*-nitrophenyl phosphate only and bis-*p*-nitrophenyl phosphate plus neostigmine before [^3H]-

Fig. 4. SDS/polyacrylamide-gel electrophoresis of enzyme fractions separated by isoelectrofocusing

The frozen fractions produced from the experiment shown in Fig. 3 (a, b, c and d) were prepared for SDS/polyacrylamide-gradient-gel electrophoresis as described in the Materials and methods section by repeated concentration and dilution in Minicon microconcentrators to remove the Ampholines. The final concentrated samples were resuspended in SDS sample buffer and electrophoresed on SDS/polyacrylamide-gradient-gels as indicated in Fig. 1 legend. (a), (b), (c) and (d) refer to SDS/polyacrylamide-gradient gels of the peak fraction indicated in Fig. 3, and I and II to the 80000- M_r and 58000- M_r subunits respectively. A densitometer scan and schematic representation of each gel is also given.

DiPF labelling (Fig. 5). These experiments showed that the labelling of peak d was completely abolished with bis-*p*-nitrophenyl phosphate alone. The same inhibitor decreased the labelling of peaks a and b by 90%. The remaining radioactivity correlated with the cholinesterase activity, and on SDS/polyacrylamide-gel electrophoresis gave a single peak at M_r 80000. The [^3H]DiPF-labelling of this band was completely inhibited by neostigmine. The peak at pI 5.0 (c in Figs. 3, 4 and 5) had a subunit M_r 58000 and was unaffected by bis-*p*-nitrophenyl phosphate and neostigmine.

Discussion

The results described in this study show that guinea-pig serum contains two separate carboxyl-

esterase enzymes. One enzyme (referred to below as carboxylesterase I) has pI 3.9, and the other enzyme (carboxylesterase II) pI 6.2. The two enzymes have not only different charge characteristics but also different subunit compositions, as determined by [^3H]DiPF-labelling experiments and SDS/polyacrylamide-gel electrophoresis. Thus carboxylesterase I is composed of 80000- M_r subunits, whereas carboxylesterase II has smaller subunits of M_r 58000. Both enzymes as determined by the inhibition of [^3H]DiPF labelling are sensitive to bis-*p*-nitrophenyl phosphate (a specific carboxylesterase inhibitor) and to paraoxon (B-esterase inhibitor) but insensitive to neostigmine (a specific inhibitor of cholinesterase). The subunit M_r values of the two enzymes may be considered in relation to previously published data on rat serum carboxyl-

Fig. 5. Isoelectrofocusing and SDS/polyacrylamide-gel electrophoresis of partially purified enzyme preparation labelled with $[^3\text{H}]\text{DiPF}$ and the effects of inhibitors on this labelling

The Figure shows the results of three experiments. In experiment 1, 1.4 mg of the partially purified enzyme was preincubated at 37°C for 30 min before labelling with $1\mu\text{M}$ - $[^3\text{H}]\text{DiPF}$ for 5 min as described in the Materials and methods section. The unchanged inhibitor was removed by Sephadex G-25 chromatography and the labelled enzyme preparation co-isoelectrophoresed with 30.3 mg of unlabelled partially purified enzyme preparation. The ^3H -radioactivity elution profile (cf. Fig. 3) is shown in the top part of the Figure (O). Experiment 2 was identical with experiment 1, except that the preincubation step was performed with 1 mM-bis-*p*-nitrophenyl phosphate (BNPP), and is shown as Δ . Experiment 3 was as experiment 2 except that neostigmine (final concn. 1 mM) was also added to the preincubation mixture, and is shown as $\blacktriangle$. The indicated fractions (cf. Fig. 3) were pooled and analysed by SDS/polyacrylamide-gel electrophoresis as described in Fig. 4 legend. In the case of fraction *a,b* the combination of neostigmine and BNPP totally inhibited $[^3\text{H}]\text{DiPF}$ labelling, and this fraction was not analysed by SDS/polyacrylamide-gel electrophoresis. Similarly, the $[^3\text{H}]\text{DiPF}$ labelling of fraction *d* was totally abolished by both inhibitors, and was therefore not electrophoresed. I and II refer to the 80 000- M_r and 58 000- M_r subunits respectively.

esterase. Choudhury (1974) reported a purified carboxylesterase that was active and had M_r 43 000 by gel filtration. In contrast, Hashinotsume *et al.* (1978) reported a highly purified carboxylesterase with M_r 84 000 (gel filtration) and pI 4.4. The latter would seem to be similar to carboxylesterase I reported in the present study. However, preliminary gel-filtration experiments on Sephadex G-200 with active serum (results not shown) showed that the carboxylesterase activity was eluted as a single broad peak with M_r in the range 150 000–200 000. This means that the enzymes, although separable by charge (Fig. 3), cannot be distinguished on the basis of size, and furthermore that the active enzymes must be oligomeric. A possible subunit arrangement would be that carboxylesterase I is a dimer (M_r 160 000) and carboxylesterase II is a trimer (M_r 190 000); if this were true, then these proteins would not be separable on Sephadex G-200. The guinea-pig enzymes are thus different from those found in rat serum, which are probably monomers. In this respect it is significant that the liver carboxylesterases are easily dissociated into their monomeric forms by high dilution, high salt concentration and low pH (see, e.g., Miller *et al.*, 1980). Thus it is possible that the purification procedures of Choudhury (1974) and Hashinotsume *et al.* (1978) dissociated the oligomeric proteins into their constituent subunits, which were also active.

The multiple forms of liver carboxylesterases are considered to be due to minor modification of charged groups on the subunits rather than to intrinsic subunit differences (Junge & Krisch, 1975). These conclusions are invariably based on 'pure' enzymes, the preparation procedures of which are biased towards high specific activity rather than total recoveries. Arndt *et al.* (1973), for example, purified a rat liver carboxylesterase (E1) that represented 18% of the original microsomal activity, and in the procedure of Haugen & Suttie (1974) the pure enzyme accounted for 16% of the original starting materials. Poor recoveries can occur in any enzyme purification procedure, and may be due to a variety of reasons, such as denaturation of the enzyme. Alternatively, a poor enzyme recovery may be indicative that the original starting material contains more than one enzyme and that the particular preparative procedures employed have selectively purified only one of the enzymes. This may explain why Haugen & Suttie (1974) found that their enzyme was a dimer of 70 000- M_r subunits, whereas

Arndt *et al.* (1973) reported a trimer of 60 000- M_r subunits. It follows, therefore, that it is of great importance to be able to characterize the enzyme population in the starting material (e.g. liver microsomal fraction or serum) before attempting purification. The present study highlights the advantages of such an approach, which can be readily applied to all B-esterases.

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