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PURIFICATION AND PARTIAL CHARACTERIZATION OF RAT INTESTINAL CEFUROXIME AXETIL ESTERASE

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Abstract—An esterase which hydrolyses the cephalosporin antibiotic, cefuroxime axetil has been isolated from rat intestinal washings and purified. Closely related cefuroxime esters were extremely poor substrates, but *p*-nitrophenyl acetate and α-naphthyl acetate were slowly hydrolysed by the purified enzyme. Analysis by gel filtration gave an M_r = 51,000 and on SDS-polyacrylamide gel electrophoresis the esterase resolved into two main bands of M_r = 31,500 and 26,800. Analytical isoelectric focusing resolved purified esterase into multiple forms active toward α-naphthyl acetate, the isoelectric points of which ranged from pH 4.5 to 6.3. The esterase bound specifically to Con A-Sepharose suggesting it could be a glycoprotein. Esterase activity was unaffected by the presence of dihydroxy bile salts (1–8 mM) and inhibition studies using organophosphates and eserine salicylate have classified the enzyme as a carboxylesterase.

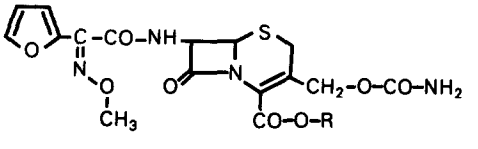
Oral administration of cefuroxime axetil (the 1-acetoxyethyl ester of cefuroxime—Fig. 1; R = R^3) in the rat results in blood levels of cefuroxime which indicate that the prodrug is about 50% absorbed. Unabsorbable cefuroxime remains in the lumen of the intestine which indicates hydrolysis of the axetil ester by an intestinal esterase. Non-specific esterases have been found in the mucosal epithelium of human [1] and rat [2, 3] small intestine and they have been resolved electrophoretically into multiple molecular forms. Although the hydrolysis of several prodrug antibiotic esters by intestinal esterases has been reported [4–6] only limited investigations have been undertaken to characterize the enzymes involved and, despite their biochemical significance, very few esterases which hydrolyse foreign compounds have been purified. This paper describes the purification and partial characterization of a carboxylesterase, isolated from rat intestinal washings, that hydrolyses cefuroxime axetil (CA).† A limited comparison with the corresponding human and dog cefuroxime axetil esterase (CAE) has been made.

MATERIALS AND METHODS

Materials and equipment. CRM(X) whole diet was purchased from Labsure Ltd. (Poole, U.K.). Biochemicals were obtained from Sigma Chemical Co. Ltd. (Poole, U.K.) and diisopropyl fluorophosphate from Aldrich Chemical Co. Ltd. (Gillingham, U.K.). Pharmacia Ltd. (Milton Keynes, U.K.) supplied Blue Dextran, Sephadex G-75 (Superfine), and Con A-Sepharose, Whatman LabSales Ltd. (Maidstone, U.K.) DEAE-cellulose (type DE52), and Bio-Rad Laboratories Ltd. (Watford, U.K.) hydroxylapatite (Bio-Gel HTP). Ultrogel AcA34 and Ampholine were obtained from LKB Instruments Ltd. (South Croydon, U.K.). Electrophoresis reagents (Electran), ammonium sulphate (ARISTAR) and other chemicals were supplied by BDH Chemicals Ltd. (Dagenham, U.K.). All purifications were performed on the LKB 2111 Multirack fraction collector and electrophoresis was undertaken on the LKB 2117 Multiphor flat bed apparatus with an LKB 2197 power supply.

Preparation of intestinal washings

(i) *Rat.* Charles River CD male rats (300–500 g) fed CRM(X) whole diet and water *ad libitum*, were fasted overnight before being anaesthetized (intraperitoneal injection of Sagatal—1 ml.kg^{-1}). An abdominal mid-line incision exposed the intestine which was cannulated 2 cm distal to the entrance of



R^1 = CH(CH$_3$)–O–CO–C(CH$_3$)$_2$–O–CH$_3$

R^2 = CH(CH(CH$_3$)$_2$)–O–CO–CH$_2$–O–CH$_3$

R^3 = CH(CH$_3$)–O–CO–CH$_3$

Fig. 1. The chemical structure of cefuroxime axetil (R^3) and related esters.

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† Abbreviations used: CA, cefuroxime axetil; CAE, cefuroxime axetil esterase; PNPA, *p*-nitrophenyl acetate; DFP, diisopropyl fluorophosphate; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate; IEF, isoelectric focusing.

the bile duct and 2 cm proximal to the ileocaecal junction according to Doluisio *et al.* [7]. The cannulated intestine was washed through with isotonic saline (10–15 ml) and the washings collected before fresh saline (15–20 ml) was left inside the intestine for 20 min. The intestines were evacuated and the washings were pooled, filtered to remove faecal material and lyophilized.

(ii) *Dog.* Washings from anaesthetized male beagle dogs (10 kg) were prepared as described above for rats except that the volumes used were increased in relation to body weight.

(iii) *Human.* Intestinal washings were obtained from patients at Northwick Park Hospital (Middx., U.K.) who were undergoing ileostomy. The washings were frozen within 1 hr of collection and lyophilized.

Assay of esterase activity

(i) *Cefuroxime axetil.* CAE activity was measured by the HPLC method described by Campbell and Langley [8]. One unit of enzyme hydrolyses 1 μmole CA per min under these conditions.

(ii) *p-Nitrophenyl acetate (PNPA).* A stock solution of PNPA in methanol (5.5 mM) was prepared daily. The incubation mixture comprised 50 mM sodium phosphate buffer, pH 7.0, PNPA (0.22 mM) and enzyme solution (0–100 μl) in a final volume of 1.0 ml. The reaction velocity was measured against an enzyme blank at 400 nm and 30° assuming $\varepsilon = 7900$ l.mol^{-1} cm^{-1} for *p*-nitrophenol under these assay conditions.

(iii) *α-naphthyl acetate (zymogram stain).* A modified method based on that of Young and Bitta [9] was used. A 4% (w/v) solution of α-naphthyl acetate in methanol (1.0 ml) was diluted to 60 ml with 0.2 M sodium phosphate buffer, pH 7.0 containing fast blue B salt (0.05%, w/v) and filtered immediately before use. Paper prints were soaked in this solution and the excess removed before laying on the gel surface. Zymograms were allowed to develop until suitable band intensities were observed (1–20 min).

Inhibition of purified CAE. Esterase (3.3 μg) was incubated with DFP, PCMB, PMSF or eserine salicylate at concentrations of 10^{-6} M, 10^{-3} M, 10^{-3} M and 10^{-5} M respectively. Each incubation contained 50 mM sodium phosphate buffer, pH 7.0 in a final volume of 160 μl. After preincubation of enzyme with inhibitor for 1 hr at 37° the reaction was started by the addition of CA substrate solution (5 μl).

Protein determination. The procedure of Lowry *et al.* [10] was used, as modified by Miller [11]. Bovine serum albumin was used as standard and the absorbance monitored at 750 nm.

Purification of rat CAE

All manipulations were undertaken at 4° unless otherwise stated and Amicon ultrafiltration cells with PM10 membranes were used for concentration steps. Column chromatography was carried out under a constant hydrostatic pressure.

(i) *DEAE-cellulose chromatography.* Lyophilized intestinal washings from 120 rats (29.7 g) were resuspended in ice-cold 10 mM Tris–HCl buffer, pH 7.8 (2 l). The salt concentration was reduced from 0.16 M to 8 mM by dialysis against the same buffer (40 l) before loading (1.8 l) on to a DEAE-cellulose column (26 × 3.5 cm) previously equilibrated with 10 mM Tris–HCl buffer, pH 7.8. The column was washed with equilibration buffer (600 ml) followed by a 0–0.2 M KCl gradient in equilibration buffer (700 ml total). Fractions containing CAE activity were pooled, concentrated to 20 ml and dialysed overnight against 10 mM sodium phosphate buffer, pH 7.0 (450 ml).

(ii) *Ammonium sulphate fractionation.* After DEAE-cellulose chromatography the purified enzyme was fractionated with ammonium sulphate, allowing standing on ice for 1 hr between centrifugation steps. Precipitates were resuspended in, and dialysed against, 20 mM sodium phosphate buffer, pH 7.0 (1.0 ml and 2 l respectively). Fractions containing CAE activity were pooled and purified by gel filtration.

(iii) *Ultrogel AcA34 chromatography.* An Ultrogel AcA34 column (90 × 3.5 cm) was equilibrated with 0.1 M sodium phosphate buffer, pH 7.2 containing 0.1 M KCl and loaded with 5.9 ml ammonium sulphate-fractionated esterase. The column was developed with equilibration buffer. Fractions containing CAE activity were pooled, concentrated to 8.8 ml and dialysed against 2 mM sodium phosphate buffer, pH 7.0 (500 ml). The Ultrogel column was calibrated as described by Campbell and Park [12].

(iv) *Hydroxylapatite chromatography.* Dialysed, Ultrogel-purified enzyme (11.6 ml) was loaded on to a hydroxylapatite column (24 × 1.7 cm) previously equilibrated with 2 mM sodium phosphate buffer, pH 7.0. The column was washed with equilibration buffer (250 ml) and the esterase eluted with a gradient of 2–100 mM sodium phosphate buffer, pH 7.0 (700 ml total). Fractions containing CAE activity were pooled and concentrated to 4.0 ml.

(v) *Con A-Sepharose chromatography.* The hydroxylapatite-purified enzyme (4.0 ml) was loaded on to a Con A-Sepharose column (10 × 1 cm) previously equilibrated in 20 mM sodium phosphate buffer, pH 7.0 containing 0.2 M NaCl and left to interact for 1 hr. After washing with equilibration buffer (100 ml), the esterase was eluted with a 0–0.5 M α-methyl D-glucopyranoside gradient in equilibration buffer (100 ml total). Fractions containing CAE activity were pooled, concentrated and dialysed against 50 mM sodium phosphate buffer, pH 7.0.

Purification of dog and human CAE. CAE from both dog and human intestinal washings was purified by the same protocol used for the rat enzyme except that the ammonium sulphate fractionation and Con A-Sepharose chromatography steps were omitted.

Electrophoresis

(i) *SDS-polyacrylamide gel electrophoresis.* This was carried out using 7.5% gels run in the phosphate buffer system of Weber *et al.* [13] as described by Fehrnström and Moberg [14]. Gels were stained with silver according to Oakley *et al.* [15].

(ii) *Analytical IEF.* This was carried out in 5% polyacrylamide gels at 4° with pH 3.5–9.5 ampholyte essentially as described by Winter *et al.* [16]. Esterase activity was detected using the α-naphthyl acetate zymogram technique and protein stained with silver as described above.

Table 1. A summary of the purification of rat CAE

Purification step	Volume (ml)	Protein (mg)	Activity (mU)	Sp. act. ($mU.mg^{-1}$)	Purification (-fold)	Yield (%)
1. Crude enzyme	1800	1044	27,846	26.7	1	100
2. DEAE-cellulose	20	105	20,470	195	7.3	73.5
3. $(NH_4)_2SO_4$ (30–70%)	5.9	88.7	15,218	171.6	6.4	54.6
4. Ultrogel AcA34	8.8	14.5	13,114	904.4	33.9	47.1
5. Hydroxylapatite	4	2.64	7,328	2,775.8	104	26.3
6. Con A-Sepharose	4.7	1.03	3,520	3,417.5	128	12.6

(iii) *Preparative IEF.* This was carried out in 5% Sephadex G-75 (Superfine) gels at 4° for 6 hr with pH 3.5–9.5 ampholyte, as described by Winter *et al.* [17]. Esterase activity, visualized by zymogram stain. was eluted from the gel with 50 mM sodium phosphate buffer, pH 7.0, concentrated and assayed for CAE activity before being examined by analytical IEF and SDS-gel electrophoresis.

RESULTS

Purification of rat CAE

The overall purification procedure is summarised in Table 1. CAE activity was eluted from DEAE-cellulose by a salt gradient (Fig. 2) and co-chromatographed with the first of two peaks active against PNPA. When fractionated by ammonium sulphate (Table 2) CAE activity was found in two main fractions (the 30–50 and 50–70%). Rather than discard 44% of the CAE activity in favour of the high sp. act. sample both fractions were pooled which resulted in a sp. act. similar to that of the original CAE sample. Figure 3 depicts CAE activity eluting from Ultrogel AcA34 at a value of $K_d = 0.58$, corresponding to a $M_r = 51{,}000$. Again two PNPA activity peaks were evident; one co-chromatographed with CAE activity and the other eluted at $K_d = 0.68$ representing a $M_r = 32{,}000$. When CAE activity was eluted from hydroxylapatite there was no evidence for a second peak of PNPA activity (Fig. 4).

The simple 4-step procedure before Con A-Sepharose chromatography resulted in a 104-fold purification and a 26% activity yield and successfully separated CAE activity from at least one other esterase capable of hydrolysing PNPA. The separation of esterases based on the hydrolysis of CA proved to

Table 2. Ammonium sulphate fractionation of rat CAE

$(NH_4)_2SO_4$ (% satn.)	Activity (mU)	Sp. act. ($mU.mg^{-1}$)
0–30	81.8	71.9
30–50	8349.4	313
50–70	6869.3	110.7
70–100	140.4	23.2

Ground, solid $(NH_4)_2SO_4$ was slowly added to enzyme solution and supernatants with constant stirring to give the required saturation (%). After standing on ice (1 hr) the precipitates were collected by centrifugation at 70,000 *g* (10 min). Activity and protein measurements were performed only on dialysed samples.

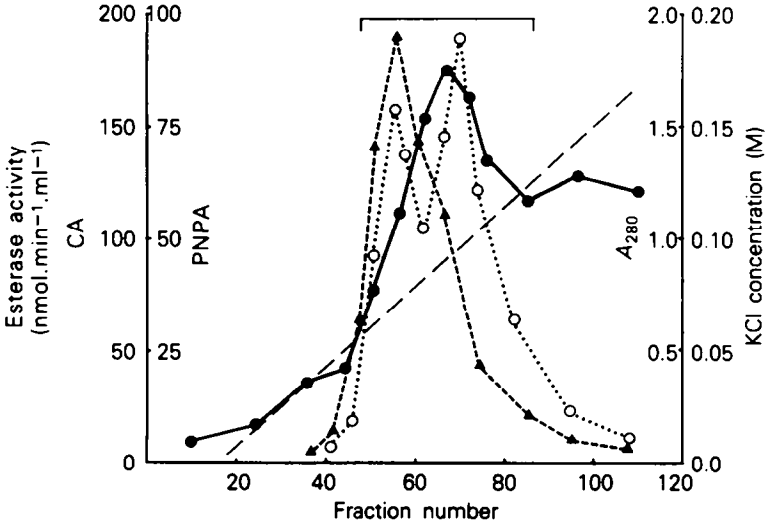

Fig. 2. Purification of rat CAE on DEAE-cellulose. Unbound protein was washed from the column with equilibration buffer, 10 mM Tris–HCl, pH 7.8, and a KCl gradient (——) in equilibration buffer was used to develop the column. Fractions enclosed within the brackets were pooled. The absorbance at 280 nm (●) and esterase activity using CA (▲) and PNPA (○) as substrates were monitored.

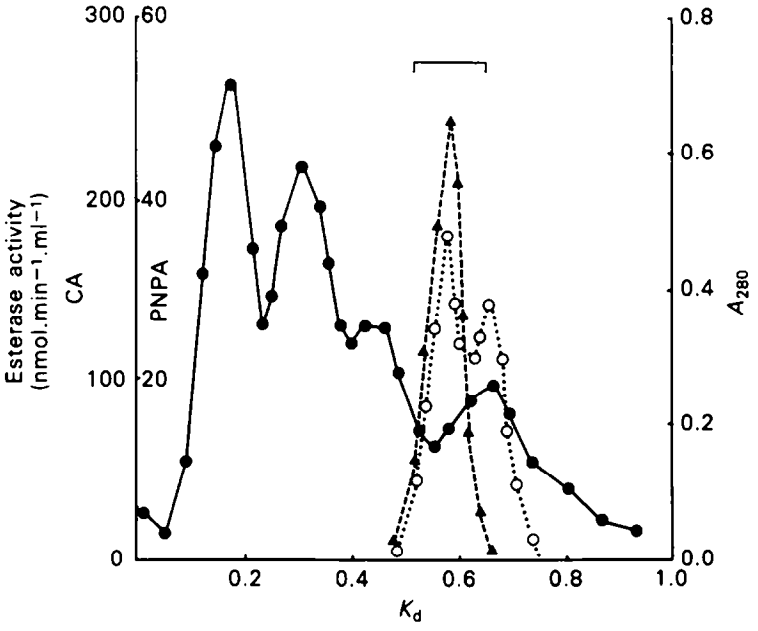

Fig. 3. Elution profile of rat CAE from Ultrogel AcA34. The column was equilibrated in 0.1 M sodium phosphate buffer pH 7.2 containing 0.1 M KCl and the absorbance at 280 nm (●) and esterase activity using CA (▲) and PNPA (○) as substrates were monitored. Fractions enclosed within the brackets were pooled. For the definition of K_d, the distribution coefficient, and details of the calibration, see Ref. 12.

be more specific than suspected because CAE activity was dissociated almost entirely, by DEAE-cellulose chromatography, from esterase activity capable of hydrolysing closely related cefuroxime esters (Table 3). Hydrolysis of the various cefuroxime esters by the same enzyme would have resulted in a constant ratio for cefuroxime production at each purification stage but no such ratio was evident.

Characterization of rat CAE

(i) *Binding to Con A-Sepharose.* Purified CAE

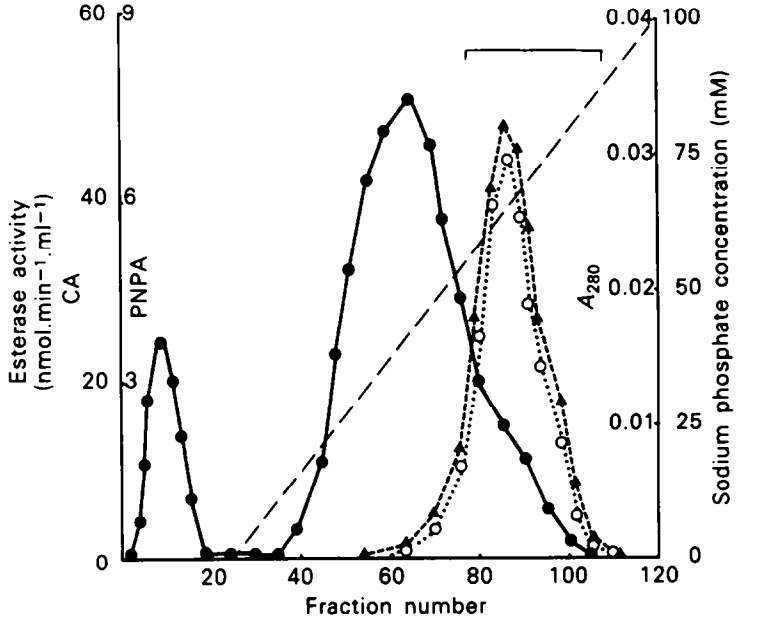

Fig. 4. Purification of rat CAE on hydroxylapatite. Unbound protein was washed from the column with equilibration buffer, 2 mM sodium phosphate, pH 7.0 and a sodium phosphate gradient (——) was used to develop the column. The absorbance at 280 nm (●) and esterase activity using CA (▲) and PNPA (○) as substrates were monitored. Fractions enclosed within the brackets were pooled.

Table 3. Rate of hydrolysis of CA and related esters by rat CAE

Purification step	Relative velocity (nmole min^{-1})		
	R^1	R^2	R^3
1. Crude enzyme	1	15	2.6
2. DEAE-cellulose	1	4.5	41.9
3. $(NH_4)_2SO_4$ (30–70%)	1	5.4	29.3
4. Ultrogel AcA34	1	5	264
5. Hydroxylapatite	1	4.8	1564

Samples of CAE, retained from each stage of the purification procedure, were assayed using as substrate cefuroxime esters (see Fig. 1) known to be de-esterified by crude gut washings at a slower (R^1) or faster (R^2) rate than CA (R^3). All substrates were incubated at a concentration of 1.78 mM and the liberated cefuroxime quantified by HPLC as described earlier. The reaction velocity with R^1 as substrate was normalized to one, and the other velocities expressed relative to this.

bound to Con A-Sepharose in the presence of 0.2 M NaCl and was eluted by 0.3 M α-methyl D-glucopyranoside suggesting that CAE was a glycoprotein. The sp. act. of CAE (Table 1) increased slightly

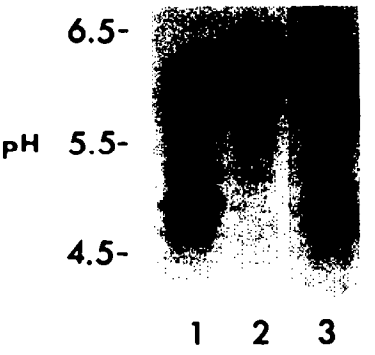

Fig. 5. A zymogram stain of hydroxylapatite-purified CAE from rat (1), human (2) and dog (3) run on a broad range (pH 3.5–9.5) analytical IEF gel.

(2.8–3.4 $U.mg^{-1}$) indicating the removal of some inactive protein but the relatively low yield (40–50%) precluded its use as a preparative step in subsequent purifications.

(ii) *Inhibition studies.* CAE was inhibited 100% by DFP (10^{-6} M) and 40% by PMSF (10^{-3} M). Eserine salicylate (10^{-5} M) failed to inhibit CAE but 15% inhibition occurred at 10^{-3} M and PCMB (10^{-3} M)

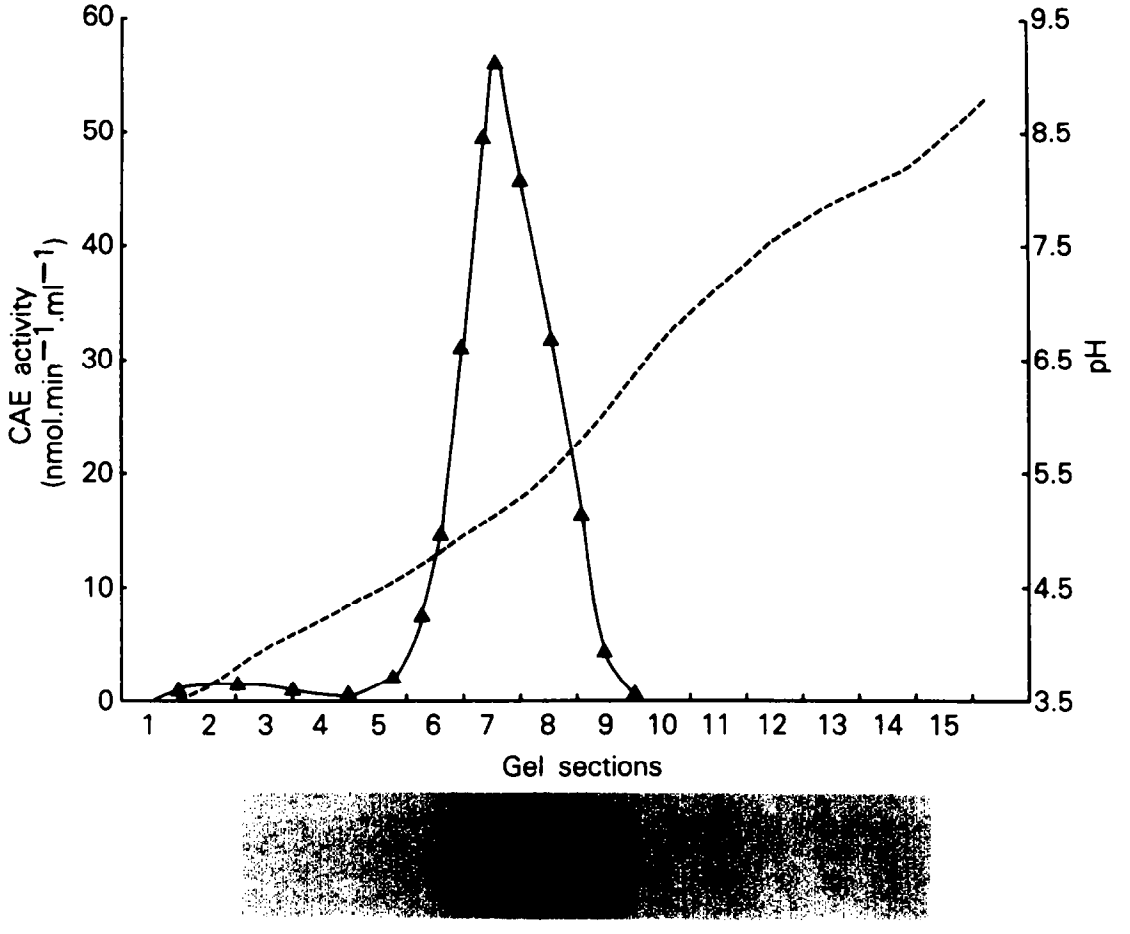

Fig. 6. A zymogram stain of hydroxylapatite-purified rat CAE (400 μg) run on a broad range (pH 3.5–9.5) preparative IEF gel. The activity (▲) of gel section eluates using CA as substrate is shown through the pH gradient (——).

did not inhibit CAE. Similarly, the multiple bands apparent on IEF and zymogram staining of CAE samples (see later) disappeared on DFP (10^{-3} M) treatment and were significantly reduced in intensity on eserine salicylate (10^{-3} M) treatment. These results suggested that the enzyme was a serine-dependent carboxylesterase.

(iii) *SDS-gel electrophoresis.* Hydroxylapatite purified CAE resolved into two main bands of M_r = 31,500 and 26,800 and a minor band at M_r = 18,300 when visualized by silver stain. No bands were detected between M_r = 50,000–60,000.

(iv) *Analytical IEF.* A typical analytical plate is shown in Fig. 5. CAE resolved into multiple discrete, but closely associated, bands. Greater than 90% of the protein that stained with silver hydrolysed α-naphthyl acetate indicating that the preparation contained very low levels of non-esterase protein. The bands all focused in the range pH 4.5–6.3 and the inhibition results described above implied that the multiple active forms were isoenzymes of the same esterase.

(v) *Preparative IEF.* Purified CAE resolved into 5 main bands, active both on zymogram staining and, after elution, with CA (Fig. 6). Active bands focused at pI values of 4.80, 5.00, 5.10, 5.25 and 5.45, the latter three bands being the most active. A poorly resolved area between pH 5.45 and 6.15 also showed some activity toward both substrates. Eluted samples of CAE, after pooling into four fractions, were either refocused on an analytical gel (Fig. 7) or run on SDS-gel electrophoresis. Refocusing results showed that the bands of activity observed on the preparative plate could be (a) resolved further into several discrete bands and (b) were probably distinct isoenzyme forms of CAE because they did not disproportionate back into the original complement of bands (although some bands were seen in more than one fraction). Refocusing of each pooled fraction in the presence of 0.1% (w/v) Triton X-100 did not decrease the number of active bands present suggesting disproportionation and/or self-aggregation were not the cause of the observed heterogeneity. SDS-gel electrophoresis of each pooled fraction resulted in an identical pattern to that observed earlier with major bands at M_r = 31,500 and 26,800 and a minor band at M_r = 18,300.

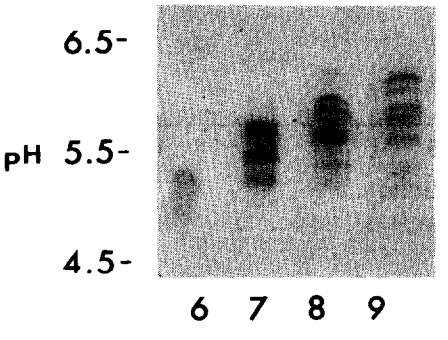

Fig. 7. A zymogram stain of fractions 6–9 (isolated from the preparative gel depicted in Fig. 6) after rerunning on a broad range (pH 3.5–9.5) analytical IEF gel.

(vi) *Effect of bile salts on CAE activity.* The sodium salts of deoxycholate, taurodeoxycholate, and glycodeoxycholate at concentrations of 1 mM and 8 mM neither activated nor inhibited CAE activity.

(vii) *Stability.* Rat CAE was stable at 1° for up to 4 weeks in 50 mM sodium phosphate buffer, pH 7.0, but both freezing and lyophilization of the purified esterase resulted in marked loss of activity. A significant loss of activity was noted on ultrafiltration.

Purification and characterization of dog and human CAE

Using a similar protocol to that described for the purification of rat CAE, dog CAE was purified only 17-fold (sp. act. = 40 mU.mg^{-1}, yield = 31%) whereas human CAE was purified 88-fold (sp. act. = 213 mU.mg^{-1}, yield = 25%). The poor purification of dog CAE may be related to the low levels of non-specific esterase found in the epithelium of the dog small intestine [4]. Dog CAE gave a M_r = 81,000 by gel filtration and on SDS-gel electrophoresis resolved into main bands of M_r = 18,400, 13,800 and 11,000 with minor bands at higher M_r values (M_r = 56,500, 35,000 and 28,000) suggesting extensive proteolysis had occurred. Human CAE gave a M_r = 51,000 by gel filtration and SDS-gel electrophoresis revealed bands at M_r = 40,300, 29,000 and 26,000. Analytical IEF resolved human and dog CAE over the range pH 4.5–6.5, although the human enzyme focused mainly at pH 5.3–6.5 (Fig. 5). Therefore, rat and dog CAE were similar regarding charge heterogeneity whereas rat and human CAE were similar regarding values of M_r.

DISCUSSION

The classification of esterases that hydrolyse xenobiotics is still in a confused state [18] but the simplistic classification based on sensitivity to organophosphates is of some practical value [19]. Cholinesterases (EC 3.1.1.7 and 8) and carboxylesterases (EC 3.1.1.1) are the major contributors to total esterase activity in the rat intestine [20] and although both are inhibited by organophosphates, carboxylesterases are insensitive (10^{-5} M), and cholinesterases are sensitive (10^{-6} M) to eserine. As rat CAE was totally inhibited by DFP (10^{-6} M) and uninhibited by eserine (10^{-5} M) it should be classified as a carboxylesterase. When classifying purified esterases, it is important to quantify the esterase activity in terms of substrate turnover. Due to extensive chemical hydrolysis of CA above pH 7.0 the pH optimum for enzymic hydrolysis could not be determined and because of the limited aqueous solubility of CA, zero-order kinetics could not be achieved. Thus a turnover number of only 200 per min was observed for CAE whereas a significantly higher value would be expected if optimal conditions could be achieved.

Different types of carboxylesterases have been described in most animal tissues, e.g. rat [20]. One of the problems encountered during their characterization is that some forms exhibit wide and overlapping substrate specificity which might be expected if they were intended to hydrolyse a wide range of exogenous esters. Inoue *et al.* [5] purified car-

boxylesterases from human hepatic tissue and intestinal mucosa and enzymes from both sources showed similar substrate specificity by hydrolysing ester-type drugs such as aspirin, clofibrate, carindacillin and procaine. Mentlein and Heymann [21] purified 5 microsomal isoenzymes from rat liver which all had high sp. act. for α-naphthyl acetate, although, one of the isoenzymes hydrolysed procaine and clofibrate, another aspirin and a third phenacetin. CAE, which was purified on the basis of its ability to hydrolyse CA, appeared quite specific in that it showed no detectable activity towards related cefuroxime esters or the aliphatic esters ethyl acetate and ethyl butyrate. Nevertheless, α-naphthyl acetate and PNPA were hydrolysed and the ratio of CA/PNPA esterase activity, for crude and pure rat CAE, was 0.2 and 6.0 respectively which confirmed that non-specific esterases had been removed during the purification. The specificity of CAE was highlighted further by a commercial porcine liver carboxylesterase preparation that showed a CA/PNPA activity ratio of only 0.003.

SDS-gel electrophoresis and gel permeation values of M_r suggested purified rat CAE comprised two non-identical subunits ($M_r = 26{,}800$ and 31,500), however this quaternary structure could be a proteolytic artifact. Values of M_r, deduced from gel filtration and SDS-gel electrophoresis studies have been reported for esterolytic activities in both rat [22–25] and man [5]; although human intestinal CAE resembled the carboxylesterase from human intestinal mucosa [5], no such similarity was evident between rat intestinal CAE and the corresponding rat enzymes [22–25].

All the protein present in purified preparations of rat CAE showed esterase activity toward α-naphthyl acetate after IEF and, although a single esterolytic reaction can frequently be mediated by several distinct enzymes, recent work with esterases and other enzymes concerned with xenobiotic metabolism has produced evidence for the existence of multiple isoenzymic forms. The heterogeneity observed in the purified rat CAE preparation implied the presence of (1) several distinct individual esterases (2) isoenzymes of a single esterase (3) aggregated species or (4) disproportionation products. As only two species were evident on SDS-gel electrophoresis of purified CAE, both before and after separation into discrete bands on preparative IEF, the heterogeneity would not appear to result from the presence of several distinct individual esterases. The complexity of the pattern was not changed by focusing on analytical gels in the presence of Triton X-100 and was independent of focusing time suggesting the heterogeneity was not a consequence of oligomeric or conformationally unfolded forms migrating at different rates to their common pI values. Also, fractions isolated from a preparative plate, failed to disproportionate into the original complement of bands when refocused on an analytical gel. Therefore, charged isomeric forms of a single esterase are probably the cause of heterogeneity and may result from differing degrees of glycosylation. Indeed CAE, by virtue of its binding to Con A-Sepharose, appeared to be a glycoprotein and the great charge diversity observed may be caused by the presence of sialic acids which are frequently found in glycoproteins of higher organisms and can be important in conferring resistance to proteolytic degradation as has been demonstrated for a variety of glycoprotein molecules from gastric mucus cells [26]. By analogy, CAE might be protected by sialic acids from extensive proteolysis in the intestinal lumen and could explain why the human and dog CAE exhibited a similar degree of charge heterogeneity. Purified carboxylesterases from rat [23, 25] and man [5] exhibit pI values in the same range as rat, human and dog CAE although CAE appears more heterogeneous regarding charge. Negrel *et al.* [3], using solubilized homogenates of rat jejunum, found approximately 14 bands active toward α-naphthyl acetate on gel electrophoresis demonstrating the numerous esterases present in rat intestine. However, only a few esterases were found to be active against esters of *p*-nitrophenol. CAE falls into the latter category.

Although bile salts are found in rat intestinal lumen at a concentration of 2–10 mM during lipid absorption such levels of bile salts had no effect on purified rat CAE activity. This is in contrast to the inhibition noted for rat pancreatic lipase and intestinal glycerol-ester hydrolase [22] and the activation of rat pancreatic carboxylesterase [23] and rat mucosal esterase [27].

The rat CAE activity purified and partially characterized does not correspond with any rat intestinal carboxylesterases described in the literature and may, therefore, represent a newly identified esterase.

REFERENCES

1. M. H. Floch, S. Van Noorden and H. M. Spiro, *Gastroenterology* **52**, 230 (1967).
2. B. Friedman, D. S. Strachan and M. M. Dewey, *J. Histochem. Cytochem.* **14**, 560 (1966).
3. R. Negrel, G. Serrero, V. Fernandez-Lopez and G. Ailhaud, *Eur. J. Biochem.* **71**, 249 (1976).
4. H. Shindo, K. Fukuda, K. Kawai and K. Tanaka, *J. Pharmacobio-Dyn.* **1**, 310 (1978).
5. M. Inoue, M. Morikawa, M. Tsuboi, Y. Ito and M. Sugiura, *Jap. J. Pharmac.* **30**, 529 (1980).
6. M. Morikawa, M. Inoue, M. Tsuboi, T. Shomura, S. Someya, S. Murata, K. Umemura and M. Sugiura, *J. Pharmacobio-Dyn.* **5**, 314 (1982).
7. J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita and J. V. Swintosky, *J. Pharm. Sci.* **58**, 1196 (1969).
8. C. J. Campbell and C. Langley, *Xenobiotica* **15**, 1011 (1985).
9. C. W. Young and E. S. Bittar, *Cancer Res.* **33**, 2692 (1973).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. G. L. Miller, *Analyt. Chem.* **31**, 964 (1959).
12. C. J. Campbell and M. V. Park, *Int. J. Biochem.* **16**, 305 (1984).
13. K. Weber, J. R. Pringle and M. Osborn, *Meth. Enzm.* **26**, 3 (1972).
14. H. Fehrnström and U. Moberg, *LKB Application Note* 306 (1977).
15. B. R. Oakley, D. R. Kirsch and N. R. Morris, *Analyt. Biochem.* **105**, 361 (1980).
16. A. Winter, K. Ek and U-B. Andersson, *LKB Application Note* 250 (1977).

17. A. Winter, H. Perlmutter and H. Davies, *LKB Application Note* 198 (1980).
18. C. H. Walker and M. I. Mackness, *Biochem. Pharmac.* **32**, 3265 (1983).
19. W. N. Aldridge, *Biochem. J.* **53**, 110 (1953).
20. R. S. Holmes and C. J. Masters, *Biochim. biophys. Acta* **146**, 138 (1967).
21. R. Mentlein and E. Heymann, *Biochem. Pharmac.* **33**, 1243 (1984).
22. V. Fernandez-Lopez, G. Serrero, R. Negrel and G. Ailhaud, *Eur. J. Biochem.* **71**, 259 (1976).
23. C. Erlanson, *Scand. J. Gastroenterol.* **10**, 401 (1975).
24. B. J. P. De-Jong, C. Kalkman and W. C. Hülsmann, *Biochim. biophys. Acta* **530**, 56 (1978).
25. E. Heymann and R. Mentlein, *Meth. Enzm.* **77**, 333 (1981).
26. R. Schauer, *Trends biochem. Sci.* **10**, 357 (1985).
27. P. M. Mathias, J. T. Harries, T. J. Peters and D. P. R. Muller, *J. Lipid Res.* **22**, 829 (1981).