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Multiple forms of sheep serum A-esterase activity associated with the high-density lipoprotein

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Five lipoproteins of sheep serum expressing A-esterase activity, but with differing activities towards four organophosphate substrates, were separated by a combination of gel filtration and ion-exchange chromatography. Each had an M_r of approx. 360000 and contained a major peptide of M_r 28000–30000 that appeared to be present as several isoforms on urea/agarose isoelectric focusing. In every case this peptide split into a number of bands on urea/agarose isoelectric focusing. The bands appear to represent isoforms of the peptide, and four lipoproteins yielded characteristic patterns of bands. This peptide resembles the apolipoprotein A-I of human serum, and available evidence suggests that this is the protein that expresses A-esterase activity. Evidence is presented for the existence of different species of high-density lipoprotein HDL_2 particles containing different complements of peptide isoforms and expressing contrasting substrate specificities towards organophosphates.

INTRODUCTION

In 1953 Aldridge [1,2] defined two classes of esterases according to their interaction with organophosphate anticholinesterases. A-esterases hydrolyse such substances whereas B-esterases are inhibited by them. The active forms of organophosphorus pesticides are inhibitors of B-esterases and are in many cases deactivated by A-esterases [3]. Mammals tend to have high A-esterase activity in the blood and the liver, and this is evidently an important factor in determining their relatively low susceptibility to such organophosphates as diazinon and pirimiphos-methyl [4,5]. By comparison birds have very low A-esterase activity and are relatively susceptible to these two compounds.

Much of the A-esterase activity towards paraoxon (paraoxonase) in the serum of sheep and humans is associated with high-density lipoprotein (HDL) [6]. In humans a number of pathological states are known to be associated with serum A-esterase activity. Serum paraoxonase activity is low in individuals who have suffered myocardial infarction [7]. The serum paraoxonase activities and the serum concentrations of HDL cholesterol and apolipoproteins A-I and A-II of patients with 'fish-eye' disease were found to be only 10% of the mean values for control subjects [8]. Recently a genetic link has been established between serum paraoxonase and cystic fibrosis in families afflicted by this disease [9,10].

Despite their importance little is known of the biochemical properties of serum A-esterases. These enzymes are currently classified as arylesterases (EC 3.1.1.2), but recent evidence suggests that different enzymes are responsible for A-esterase and arylesterase activities in the serum of sheep and humans [11]. Studies with sheep serum have produced strong evidence for the existence of multiple forms of A-esterase with overlapping substrate specificities [12]. The present study is concerned with the further characterization of the A-esterases of sheep serum by the use of four different organophosphate substrates.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical-reagent grade unless otherwise stated. Sucrose, acetic acid, KBr, urea, $CaCl_2$, methanol (Spectrograde), NaCl and Whatman DE-52 ion-exchange resin were obtained from Fisons. Tris (Sigma 7–9 Trizma biochemical buffer), acrylamide and *NN'*-methylenebisacrylamide (both especially pure for electrophoresis), *NNN'N'*-tetramethylethylenediamine, ammonium persulphate, 2-mercaptoethanol, Bromophenol Blue, glycine and Coomassie Brilliant Blue R-250 were obtained from Sigma (London) Chemical Co. Sepharose 6-B gel-filtration medium was from Pharmacia. Isogel agarose-EF and Ampholines pH 4–7 were obtained from LKB.

Substrates

Paraoxon (*OO*-diethyl *O*-*p*-nitrophenyl phosphate [substrate (I); see Fig. 1] was purchased from Sigma. Diazoxon [*OO*-diethyl *O*-2-(1-methylethyl)-4-pyrimidinyl phosphate (II)], chlorpyrifos-oxon [*OO*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphate (III)] and coroxon [*OO*-diethyl *O*-3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-6-yl phosphate (IV)] were kindly given by CIBA-GEIGY (Whittlesford, Cambs., U.K.), Dow Chemical Co. (Wantage, Oxon., U.K.) and Wellcome Laboratories (Berkhampsted, Herts., U.K.) respectively. All substrates had a purity of 99.5% or greater.

Sheep serum

Adult female sheep blood was obtained unheparinized from Reading Abattoir and centrifuged at 2100 *g* for 1 h at 4 °C to obtain the serum.

Abbreviation used: HDL, high-density lipoprotein.

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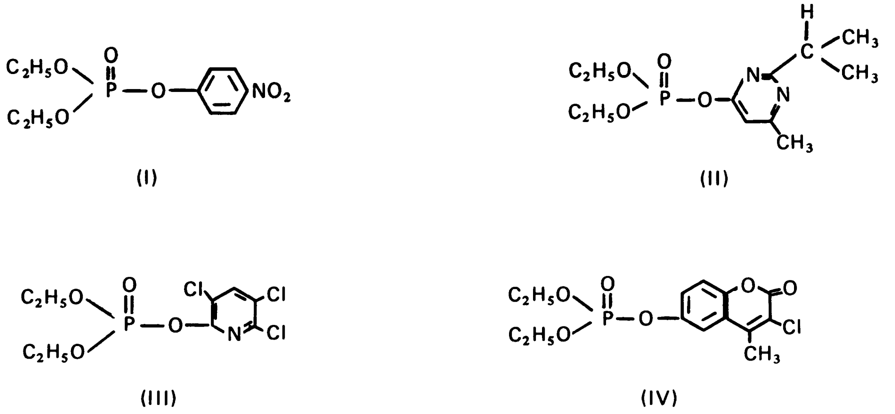

Fig. 1. Chemical structures of paraoxon (I), diazoxon (II), chlorpyrifos-oxon (III) and coroxon (IV)

Purification of A-esterase

All procedures were performed at 4 °C. The initial stage of the purification was the preparation of an HDL fraction from serum as described previously [12]. A 12 ml portion of the HDL fraction was made 5 % (w/v) with respect to sucrose and applied to a 4.5 cm × 30 cm column (Amicon) of Sepharose 6B gel-filtration medium equilibrated with 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-$CaCl_2$. The column was eluted with the same buffer at a flow rate of 20 ml/h, and 5 ml fractions were collected with an LKB 2112 Redirac fraction collector. Individual fractions were assayed for A-esterase activity towards substrates (I) and (II) and for protein.

Individual enzyme peaks from the gel filtration (see the Results section) were further purified by ion-exchange chromatography on Whatman DE-52 anion-exchange resin equilibrated in 20 mM-Tris/HCl buffer, pH 8.2 (buffer A), and packed into 1.1 cm × 10 cm columns (Amicon). The flow rate used throughout the procedure was 1.5 ml/min. Pooled fractions from gel filtration were diluted to 20 ml with buffer A and applied to the column. The column was then washed with 20 ml of buffer A followed by 20 ml of buffer A containing 1 mM-$CaCl_2$ and finally 10 ml of buffer A containing 1 mM-$CaCl_2$ and 0.1 M-NaCl. The bound enzyme was then eluted from the column by using a linear salt gradient of 0.1–0.5 M-NaCl in buffer A containing 1 mM-$CaCl_2$. During the washing procedure 4 ml fractions were collected, and 1 ml fractions were collected during the gradient elution into tubes containing sufficient $CaCl_2$ to give a final concentration of 5 mM in each fraction. Individual fractions were assayed for paraoxonase activity and protein.

Assay of enzyme activity

Paraoxon. A-esterase activity towards paraoxon (paraoxonase) was assayed by the method described by McElveen *et al.* [7].

Diazoxon (II), chlorpyrifos-oxon (III) and coroxon (IV). Enzyme samples were preincubated for 2 min in 20 mM-Tris/HCl buffer, pH 7.6, containing 10 mM-$CaCl_2$ (to give a final volume of 1 ml) at 37 °C before the addition of 20 μl of 0.1 M substrate in methanol, to give a final concentration of 2 mM. Incubation was continued at 37 °C. After addition of substrate, 200 μl portions of the assay medium were removed at 5 min and 10 min intervals for substrates (II) and (III) and at 15 min and 30 min intervals for substrate (IV), and the reaction was stopped by mixing them with 800 μl of ice-cold methanol. Precipitated protein was removed by centrifugation at 11600 *g* in an MSE microcentrifuge for 10 min, and the clear supernatant was injected on to an h.p.l.c. column.

Separation of substrates from hydrolysis products was achieved by reverse-phase h.p.l.c. on a 12.5 cm S5ODS-Spherisorb column (Hichrom, Reading, Berks., U.K.) by using a modification of the method developed by Brealey & Lawrence [13]. Elutions were performed isocratically with methanol/0.1 M-sodium phosphate buffer, pH 4.5 (3:2, v/v), with the use of a single-stroke pump (made by Metering Pumps) at 13.5 MPa (1500 lbf/in^2) and at a flow rate of 2.2 ml/min. U.v. absorbances were measured at 230 nm [substrate (II) and its product], 320 nm [substrate (III) and its product] and 310 nm [substrate (IV) and its product] with a Cecil 212E variable-wavelength u.v. spectrophotometer fitted with a 1 cm-light-path flow cell, and peak heights were measured.

Under these conditions formation of product is linear with time and protein concentrations for up to 30 min for substrates (II) and (III) and 60 min for substrate (IV) and with up to 3 mg of protein per assay.

Protein determinations

The protein contents of fractions from the column chromatographic separations were monitored by measuring the absorbance at 280 nm; all other protein determinations were carried out using the method of Bradford [14] with bovine serum albumin as standard.

Analytical polyacrylamide-gel electrophoresis

Analytical denaturing polyacrylamide-gel electrophoresis was carried out by the method of Laemmli [15] adapted for vertical slab-gel electrophoresis. An 11 % (w/v) polyacrylamide gel was overlaid with a 3 % (w/v) stacking gel. Electrophoresis was at 20 mA for 4 h, after which the gel was stained for protein in 0.1 % Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (45:9:46, by vol.) and destained in methanol/acetic acid/water (5:1:5, by vol.) to remove background staining.

Urea/agarose isoelectric focusing

Analytical isoelectric focusing in the presence of 6 M-urea was as described by Olsson & Laas [16]. Gels were prefocused for 15 min at 1500 V to allow the formation of the pH 4–7 gradient, before the addition of 20 μg of protein dissolved in 10 mM-Tris/HCl buffer, pH 8.2, containing 7 M-urea, and focusing for 1 h in a water-cooled LKB 2117 Multiphor II electrophoresis unit. After the focusing, proteins were fixed and stained as described by Olsson & Laas [16].

RESULTS

The elution profiles of protein and A-esterase activity from gel filtration of the HDL fraction of serum are shown in Fig. 2. The A-esterase activity, as measured by diazoxon hydrolysis, was found in two major peaks and one minor peak. Coroxon hydrolysis had a similar profile to diazoxon hydrolysis (results not shown). Accordingly fractions 36–40, 42–47 and 49–60, which represented these peaks, were pooled as fractions GI, GII and GIII respectively, and further purified by ion-exchange chromatography.

Ion-exchange chromatography of fraction GI led to the separation of two peaks showing paraoxonase activity (Fig. 3*a*). The fractions containing this activity were pooled and labelled IEC (1) and IEC (2) respectively. Two peaks of paraoxonase activity were also evident after ion-exchange chromatography of gel-filtration fraction GII (Fig. 3*b*), and these were pooled as IEC (3) and IEC (4). Ion-exchange chromatography of gel-filtration fraction GIII (Fig. 3*c*) yielded one peak of paraoxonase activity, which was designated as IEC (5).

The pooled fractions IEC (1) to IEC (5) were then further assayed for protein and A-esterase activity towards diazoxon (II) and chlorpyrifos-oxon (III) (Table 1). When (II) and (III) were used as substrates, the highest purification factors were observed in the case of fraction IEC (2) (182-fold and 848-fold respectively when compared with serum), followed by fraction IEC (4) (91-fold and 172-fold). Poor purification factors were obtained with paraoxon as substrate. The recoveries of enzyme activity after ion-exchange chromatography for each individual substrate were: substrate (I), 105%; substrate (II), 70%; substrate (III), 96%. These values refer to the final recovery of activity from the ion-exchange columns compared with the initial activity applied to the Sepharose gel-filtration column in the HDL fraction. Enzyme activity in the purified fractions was stable for 5 months on storage at −20 °C. None of the enzyme fractions hydrolysed α-naphthyl acetate, a substrate for B-esterases.

The ratios of enzyme activity, counting the specific activity towards paraoxon as 1 (Table 1 and Fig. 4), indicate that each peak represents a different A-esterase isoenzyme or complement of isoenzymes. The highest specific activity towards substrate (III) was shown by fraction IEC (2), the lowest by fractions IEC (3) and IEC (5). The highest specific activity towards substrate (II) was shown by fraction IEC (5). Fractions IEC (1) and IEC (4) showed relatively low specific activities towards both of these substrates.

Analytical denaturing polyacrylamide-gel electrophoresis (Fig. 5) indicated that all five peaks of A-esterase

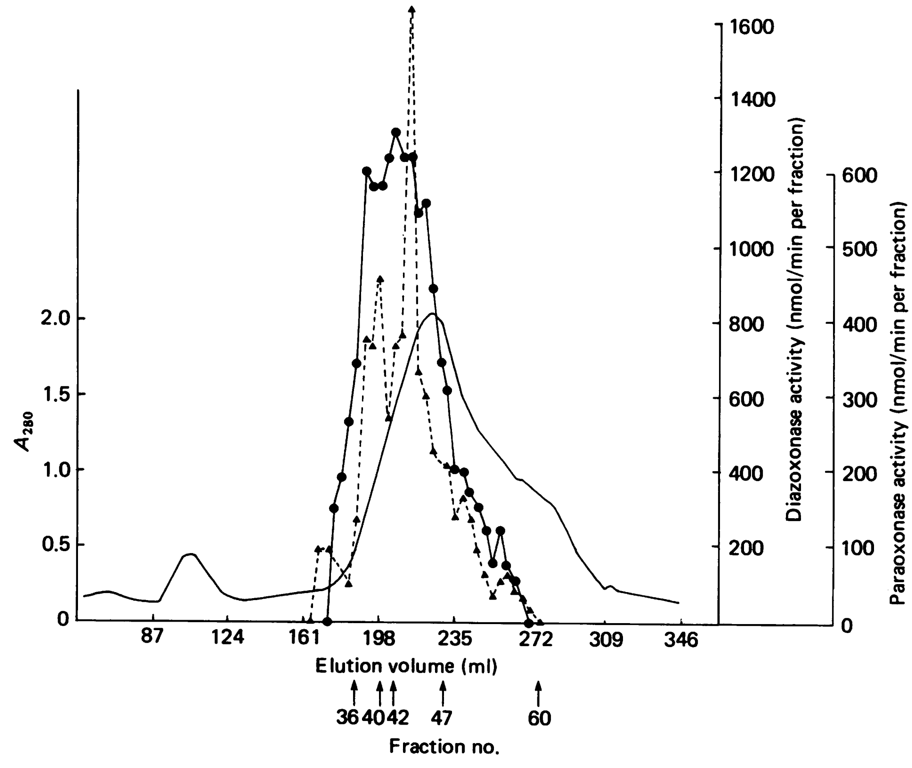

Fig. 2. Protein and A-esterase elution profiles from gel filtration of the HDL fraction of sheep serum

Details of methodology are given in the Materials and methods section. ——, Protein (A_{280}); ▲---▲, diazoxonase activity (nmol of hydroxypyrimidine produced/min per fraction); ●–●, paraoxonase activity (nmol of *p*-nitrophenol produced/min per fraction).

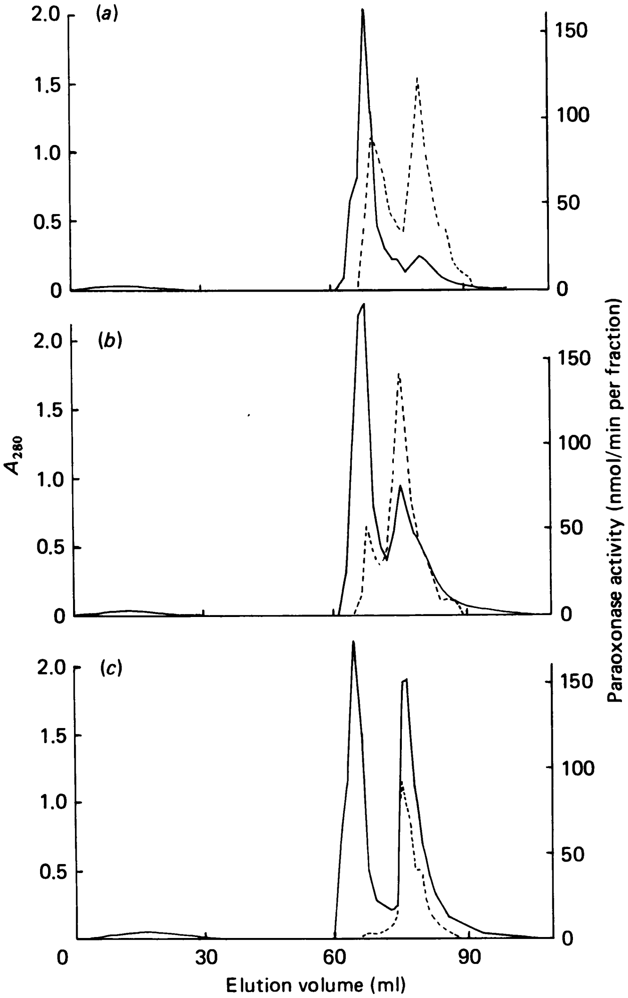

Fig. 3. Protein and paraoxonase elution profiles from anion-exchange chromatography of fractions from sheep serum HDL purified by gel filtration

(*a*) Gel-filtration fractions 36–40 (fraction GI); (*b*) gel-filtration fractions 42–47 (fraction GII); (*c*) gel-filtration fractions 49–60 (fraction GIII). Details of methodology are given in the Materials and methods section. ——, Protein (A_{280}); ------, paraoxonase activity (nmol of *p*-nitrophenol produced/min per fraction).

activity were similar in composition. The major bands in all enzyme peaks were peptides with estimated M_r values of 28000–30000. Fractions IEC (1), IEC (2) and IEC (3) exhibit the greatest degree of purity, with a single minor contaminant of M_r 66000. Double-immunodiffusion [17] against antibodies raised in rabbits have shown this contaminant to cross-react with anti-albumin antibodies (results not shown); however, it is not known whether this is the only component of this band.

Analytical isoelectric focusing in agarose gels in the presence of 6 M-urea indicated the presence of several bands with isoelectric points between pH 4.6 and pH 6.1 in all the enzyme fractions (Table 2). At least nine bands could be seen within this range with fraction IEC (5) and eight bands were present in each of fractions IEC (3) and IEC (4). Five bands were present in each of fraction IEC (1) and IEC (2) and had isoelectric points of pH 4.35, 4.45, 4.50, 4.52 and 4.57. Several of these bands were common to all the enzyme fractions (see Table 2);

Table 1. A-esterase activity in the five enzyme fractions separated by anion-exchange chromatography

Details of methodology are given in the Materials and methods section. Specific activity is defined as nmol of product produced/min per mg of protein. Original serum specific activities were 4.4 (paraoxonase), 8.9 (diazoxonase) and 11.4 nmol/min per mg (chlorpyrifos-oxonase). Recovery (%) is defined as 100 × (total activity in fraction)/(total activity in original serum). Purification factor is defined as (specific activity in fraction)/(original serum specific activity). Ratios of activities are calculated by using the specific activities and counting paraoxonase specific activity as 1. Abbreviations: CPO, chlorpyrifos-oxonase; D, diazoxonase; P, paraoxonase.

			Paraoxonase				Diazoxonase				Chlorpyrifos-oxonase				Ratio of activity	
Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Recovery (%)	Purification factor	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Recovery (%)	Purification factor	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Recovery (%)	Purification factor	CPO/P	D/P
IEC (1)	9.3	0.76	26.2±1.2	185	3.7	5.9	342±102	2420	2.4	38.5	888±185	6280	4.8	77.9	33.9	13.1
IEC (2)	10.85	0.15	146±0	237	4.7	32.9	1620±413	2640	2.6	182.1	9670±956	15700	12.2	848	66.4	11.2
IEC (3)	6.2	1.46	10.9±0.0	98.5	1.97	2.5	59.5±9.7	538	0.5	6.7	51.4±10.9	465	0.4	4.5	4.7	5.5
IEC (4)	15.5	0.44	56.5±3.2	385	7.7	12.8	805±37.8	550	5.5	90.5	1960±161	13300	10.4	172	34.7	14.3
IEC (5)	12.4	0.86	17.7±0.0	189	3.8	4.0	270±35.3	2880	2.8	30.4	49.4±3.0	527	0.4	4.4	2.8	21.8

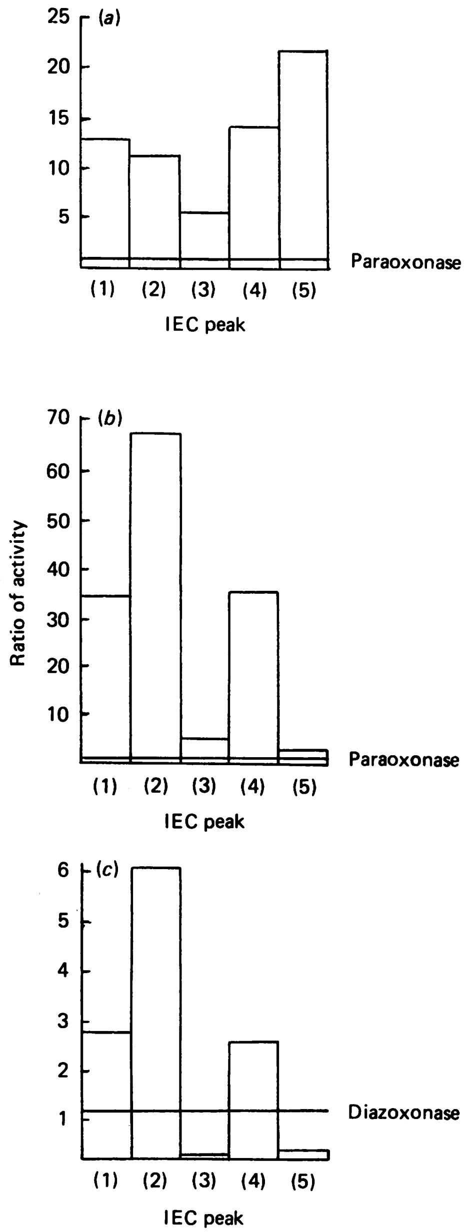

Fig. 4. Ratios of A-esterase activity in the five enzyme fractions prepared by anion-exchange chromatography

(*a*) Ratio of paraoxon to diazoxon hydrolysis (ratios calculated counting paraoxonase specific activity as 1); (*b*) ratio of paraoxon to chlorpyrifos-oxon hydrolysis (ratios calculated counting paraoxonase specific activity as 1); (*c*) ratio of diazoxon to chlorpyrifos-oxon hydrolysis (ratios calculated counting diazoxonase specific activity as 1). Details of methodology are given in the Materials and methods section.

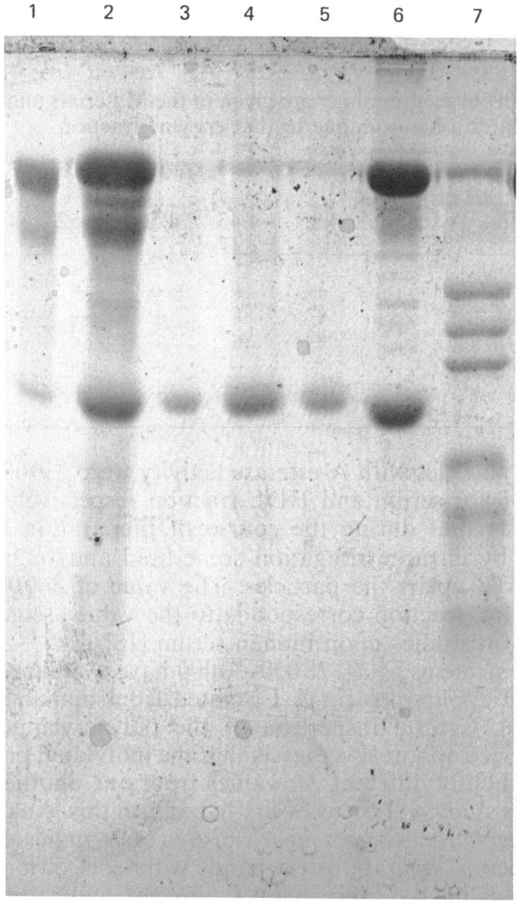

Fig. 5. Analysis by analytical denaturing polyacrylamide-gel electrophoresis of the proteins in the five A-esterase fractions produced by anion-exchange chromatography

Polyacrylamide-gel electrophoresis and protein staining are described in the Materials and methods section. Lane 1, fraction IEC (5); lane 2, fraction IEC (4); lane 3, fraction IEC (3); lane 4, fraction IEC (2); lane 5, fraction IEC (1); lane 6, sheep serum HDL; lane 7, standard protein mixture (Sigma Dalton Mark IV).

however, fractions IEC (3), IEC (4) and IEC (5) contained bands that were unique to each enzyme fraction. Which of the bands represented the 66000-M_r contaminant is, however, unknown.

DISCUSSION

These results give further evidence for the existence of multiple forms of A-esterases. Five different HDL A-esterase peaks were isolated by anion-exchange chromatography, all of which showed different substrate specificities, dramatically so in the case of fractions IEC (1), IEC (3) and IEC (5). With paraoxon (I) as substrate, low purification factors were obtained compared with substrates (II) and (III), indicating that paraoxon is a more general substrate for A-esterase isoenzymes than are the other organophosphates studied here. When these peaks were examined by polyacrylamide-gel electrophoresis they were all found to contain peptides of estimated M_r 28000–30000. Proteins of this M_r were found to be major components of purified HDL A-esterases of sheep serum in an earlier study [12]. Fractions IEC (1), IEC (2) and IEC (3) showed the highest degree of purity, with only a low degree of contamination with a protein of M_r 66000, some or all of which is albumin. When peaks IEC (1) and IEC (4) were re-run on anion-exchange chromatography, single peaks of A-esterase activity were separated in the same positions as previously, thus indicating the stability of the lipoproteins on this column system.

After gel filtration the average estimated M_r of the

Table 2. Isoelectric points of peptide bands from ion-exchange chromatography purified A-esterase fractions, separated by agarose/urea isoelectric focusing

Details of methodology are given in the Materials and methods section. +, Peptide present; −, peptide absent. An underlining indicates a band unique to that enzyme fraction.

Fraction	pI of peptide band...	4.35	4.45	4.50	4.52	4.57	4.65	4.8	5.1	5.3	5.42	5.62	5.75	5.87	6.05
IEC (1)		+	+	+	+	+	−	−	−	−	−	−	−	−	−
IEC (2)		+	+	+	+	+	−	−	−	−	−	−	−	−	−
IEC (3)		+	+	+	+	+	−	−	−	$\underline{+}$	−	+	−	−	+
IEC (4)		−	−	+	+	+	−	$\underline{+}$	$\underline{+}$	−	−	+	−	+	+
IEC (5)		−	+	+	+	+	$\underline{+}$	−	−	−	$\underline{+}$	−	$\underline{+}$	+	+

HDL particles with A-esterase activity were 230000 and 360000 for serum and HDL fraction respectively. This suggests that during the course of preparation of the HDL by ultracentrifugation some lipid and/or protein is picked up by the particles. The value of 360000 for the HDL fraction corresponds to the value assigned to HDL_2 in studies upon human serum [18].

The proteins of M_r 28000–30000 have a similar M_r to that of apolipoprotein A-I isolated from human serum [19–21]. Careful inspection of the polyacrylamide gel after electrophoresis suggests that the individual proteins have slightly different M_r values from one another, but further study will be necessary to confirm this. Analytical isoelectric focusing in agarose/urea gels indicated the presence of several protein bands with isoelectric points between pH 4.6 and pH 6.1, further indicating the heterogeneity of these peptides. Moreover, each of the A-esterase lipoproteins [fractions IEC (1)–IEC (5)] revealed a different pattern of bands after isoelectric focusing, establishing differences in peptide composition between these HDL particles.

Isoelectric focusing of purified human apolipoprotein A-I has shown the presence of at least five isoforms of the protein with pI values of 5.62, 5.54, 5.46, 5.39 and 6.36 [22,23]. Several of the protein bands from the anion-exchange chromatography fractions had pI values in this region, a further indication of similarities between these proteins and apolipoprotein A-I. It is very probable that these proteins are responsible for the A-esterase activity associated with the HDL particles, since the albumin that is there has no measurable activity. Further, the study of patients with 'fish-eye' disease [8] suggests that the apolipoprotein A-I expresses all or most of the A-esterase activity in human HDL. Very low apolipoprotein A-I contents in HDL were associated with correspondingly low A-esterase activities in these patients.

The heterogeneity of human serum HDL particles is well documented [24–26], and the HDL apolipoproteins appear to form the basis of this particle heterogeneity [25]. It therefore seems probable that the lipoproteins with A-esterase activity separated in this study represent different HDL_2 particles containing different complements of isoforms of apolipoprotein A-I.

There is, therefore, growing evidence for the existence of different isoforms of a protein similar to human apolipoprotein A-I, with contrasting substrate specificities towards organophosphates, in the HDL fraction of sheep serum. If this interpretation is correct, there are interesting parallels with the B-esterases, monooxygenases and epoxide hydrolases of the endoplasmic reticulum [27–29]. These enzymes operate against lipophilic substrates in a hydrophobic environment, and exist in a number of different isoforms with contrasting, yet often overlapping, substrate specificities. The hydrolytic activity of HDL A-esterases towards endogenous substrates has yet to be established. It is interesting to speculate that members of all these classes of hydrophobic enzymes have roles in endogenous metabolism but that particular isoforms have evolved (sometimes with very wide substrate specificity) to metabolize naturally occurring xenobiotics. In this context it may be noted that rat liver microsomal B-esterases have been shown to hydrolyse palmitoyl-CoA and monoacyl glycerols as well as a variety of ester and amide type xenobiotics [27,30,31].

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