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Electrophoresis Studies on Blood Plasma Esterases

I. Mammalian Plasmata

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The esterase activity of mammalian plasmata was investigated using as substrates a series of aliphatic, aromatic, heterocyclic and choline esters. Electrophoresis on cellulose columns was used for separation of the various esterases present in each plasma. The distribution of esterase activity and total protein after electrophoretic separation are reported for the plasmata of man, monkey, horse, cow, reindeer, goat, swine, cat, dog, guinea-pig, rabbit, and rat. Each esterase component was further studied as to substrate specificity and sensitivity to certain selective inhibitors.

Three types of esterases were present in the plasmata studied. An arylesterase (aromatic esterase) migrated on electrophoresis in most cases together with or close to the albumins, an aliesterase ("lipase") in the α_1-globulin region, and a cholinesterase between the α_2- and β-globulins. Within each group of esterases there were species variations in the properties of the individual enzymes. All plasmata studied contained an arylesterase which hydrolysed phenyl acetate at a higher rate than phenyl butyrate. Two arylesterases were found in the plasma of reindeer and rabbit. Aliesterases hydrolysed aliphatic and aromatic esters but not choline esters; phenyl acetate was hydrolysed at a lower rate than phenyl butyrate. Such enzymes were not detected in human, monkey, and dog plasmata. The concentration and properties of the plasma cholinesterases varied greatly with species. The ruminant plasmata had very low or no cholinesterase activity. Butyrylcholinesterase was present in the man, horse, cat, dog, and guinea-pig; propionylcholinesterase was found in the cow, rabbit, and rat; and acetylcholinesterase was present in the goat. Yet another type of cholinesterase occurred in swine plasma.

Differentiation of blood plasma esterases has previously been based mainly on substrate specificity and inhibitor experiment with orginal plasma (serum) or crude enzyme preparations. In the present investigation these enzymes were separated electrophoretically, the properties of the esterase fractions obtained were studied, and the esterase patterns of various vertebrate plasmata were compared.

The results, briefly summarized in a previous communication [1], showed that vertebrate plasmata contain three types of esterases, the characteristic features of which are as follows:

Arylesterases: aromatic esterases which hydrolyse phenyl acetate at a higher rate than phenyl butyrate; aliphatic esters are normally not attacked. They are resistant to certain organophosphorus compounds and to physostigmine, and have, in most cases, the greatest electrophoretic mobility of the three esterase groups.

Aliesterases, including lipases: both aliphatic and aromatic esters are hydrolysed, but not choline esters. There exist acetyl-, propionyl- and butyryl-aliesterases. They are sensitive to 10^{-5} M of a great number of organophosphorus compounds, but resistant to 10^{-5} M physostigmine; some of them are sensitive to higher concentrations of the latter compound. In most cases, these enzymes move electrophoretically more slowly than the arylesterases, but more rapidly than cholinesterases.

Cholinesterases: choline esters are split at a higher rate than both aliphatic and aromatic esters, the latter usually being hydrolysed at a lower rate than aliphatic esters or not at all; the specificity regarding the acyl group varies greatly. These esterases are sensitive to many organophosphorus compounds 10^{-6} M or less and to physostigmine, the latter giving complete inhibition in 10^{-5} M or lower concentration. They have the lowest electrophoretic mobility of all plasma esterases.

In the present and following reports, describing the experimental results on which the above classification is based, these three types of enzymes will be referred to, for practical reasons, as A-, B- and C-esterases respectively.

MATERIAL AND METHODS

Plasmata. Plasma was prepared from heparinized blood (venous or heart) and was free from all cells, including thrombocytes. Serum instead of plasma was used in some cases; both had the same esterase activity when tested with various substrates.

Substrates. The aliphatic, aromatic and choline derivatives used were the acetyl (Ac), propionyl (Pr), and butyryl (Bu) esters of the following radicals: *iso*amyl (Am), glycerol (T; *e. g.*, tributyrin, TBu), phenyl (Ph) and choline (Ch); also employed were acetyl-β-methylcholine (MeCh), benzoylcholine (BzCh), succinylcholine (SuCh), the carbon analogue of acetylcholine (3,3-dimethylbutyl acetate), Tween 20 (polyoxyethylene sorbitan monolaurate), procaine hydrochloride, atropine sulphate, cocaine hydrochloride, and heroin. Choline esters were all used in the form of iodides. The substrates were dissolved in a bicarbonate-CO_2 buffer solution (pH 7.4) to give 0.01 M in the reaction mixture.

The activity of some plasma protein fractions was also tested against indoxyl acetate. One half ml of the fractions from electrophoresis were added to 1.0 ml of a 10^{-3} M substrate solution. The intensity of indigo-blue dye formed in 30 min at 22°C was determined in these preliminary studies.

Esterase inhibitors. Eight compounds frequently used as selective enzyme inhibitors in recent esterase studies [2] were employed: physostigmine salicylate, tetra-*iso*propylpyrophosphoramide (*iso*-OMPA), *bis*-mono*iso*propylamino fluorophosphine oxide (mipafox), 10-(1-diethylaminopropionyl) phenothiazine hydrochloride (Astra 1397), 1,5-*bis*(4-trimethylammoniumphenyl) pentane-3-one diiodide (62C47), *bis*-(piperidinomethyl coumaranyl-5) ketone (3318CT), atoxyl (sodium arsanilate), and quinine sulphate. The compounds were dissolved in distilled water, and the enzyme preparations were incubated at 25°C for 50 min with the inhibitor solution before addition of substrate. The efficiency of esterase inhibition was expressed in pI_{50} values, *i. e.* negative logarithm of molar inhi-

Table 1. Enzymic hydrolysis of various esters by mammalian plasmata. Samples from at least three animals of each species were analysed. The activity values (b_{30}) refer to initial hydrolysis rates with a representative sample and are expressed in μl CO_2 per 0.10 ml of plasma per 30 min, corrections made for nonenzymic hydrolysis. Where values are indicated in brackets, initial hydrolysis rate was determined by extrapolation. An asterisk denotes that the activity varies greatly from individual to individual. Code names are explained in the text.

Species	AcCh	PrCh	BuCh	MeCh	BzCh	SuCh	AmAc	AmPr	AmBu	TAc	TPr	TBu	PhAc	PhPr	PhBu
Man	135	310	360	2	50	3	10	15	50	5	12	55	3 300	1 000	260
Monkey (Macaque	22	44	71	1	9	1	2	3	6	2	6	10	655	200	50
Monkey Mangabey)	180	390	540	2	70	8	8	25	75	10	65	90	225	290	395
Horse	130	225	365	2	40	1	25	40	70	25	245	100	2 200	650	310
Cow	3	4	2	0	0	0	4	4	3	1	2	4	4 700	600	100
Reindeer	8	14	15	<1	2	0	1	2	4	2	5	4	250	100	50
Goat	8	7	2	2	<1	<1	2	1	2	2	4	2	5 600	590	160
Sheep	7	7	2	2	<1	<1	2	2	2	2	3	2	5 500	550	150
Swine	16	14	23	1	3	2	4	1	2	<1	4	3	450*	200*	25*
Cat	50	75	150	5	12	1	6	8	40	45	260	80	5 700	1 250	500
Dog	70	115	180	6	30	0	4	<1	6	3	10	12	2 800	600	240
Guinea-pig	50	130	170	5	20	0	(150)	(200)	(30)	200	530	530	1 700	2 130	1 200
Rabbit	16	16	10	4	2	0	4	6	15	20	40	50	7 500*	2 400*	650*
Rat	20	30	15	5	10	1	(50)	(85)	(90)	45	150	90	3 000	1 700	1 150

Table 2. Susceptibility of plasma esterases to various inhibitors. A, B and C are the esterase types referred to in the text. Values refer to negative logarithm of molar inhibitor concentration producing 50 % esterase inhibition. They were obtained in experiments with plasma and/or

Species	Physostigmine			*iso*-OMPA			Mipafox			Astra 1397		
	A	B	C	A	B	C	A	B	C	A	B	C
Man	<3	—	7.7	<3	—	6.3	<3	—	6.7	<3	—	5.6
Monkey *	<3		8.5	<3		8.0	<3		8.0			≈6
Horse	<3	<3	8.0	<3	<3.5	7.7	<3	5.7	7.5	<3.5	<3.5	5.7
Cow	<3	<3	7.3	<3	≈6	≈6	<3	≈6	≈6	<3	<3	<3.5
Reindeer	<3	—	7.2	<3	—	7.6	<3	—	7.5	<3	—	5.2
Goat	<3		7.5	<3		3.8	<3		<4	<3		<3
Swine **	<3.5		7.0	<3		6.5	<3.5		6.5	<3		4.1
Cat	<3	≈3.5	7.8	<3	≈7	7.2	<3.5	≈8	7.7	<3.5	<3.5	5.8
Dog ***	<3		8.5	<3		7.7	<3		7.5	<3		6.2
Guinea-pig ****	<3	<3	8.5	<3	5.2	7.0	<3	7.9	7.0	<3	<3	6.2
Rabbit	<3.5	<3	6.4	<3.5	6.9	<4	<3	5.6	4.2	<3.5	<3	<3.5
Rat	<3.5	<3	6.3	<3.5	5.8	6.7	<3	7.4	6.4	<3.5	<3	6.6

* Values obtained with a plasma sample from macaque monkey.
** Values obtained with a plasma having high activity (b_{30} 300) against phenyl acetate.

bitor concentration producing 50 % enzyme inhibition; these values were obtained by extrapolation from curves of inhibition (%)-inhibitor concentration (pI), where sufficient inhibitor was used at the higher pI levels to establish the point of complete inhibition. The active fractions obtained in electrophoresis experiments were dialysed against the bicarbonate buffer prior to testing their sensitivity to inhibitors.

Electrophoresis. Preparative column electrophoretic separation was performed with an apparatus described by Svensson [3] using cellulose powder. ("Munktell's cellulose powder", prepared according to the direction of Flodin and Kupke [4]). Electrophoretic runs were carried out in veronal buffer solution (pH 8.4, $I = 0.1$) at 5—11°C as maintained by a water-cooled jacket. Columns of two sizes were used, 3 cm × 40 cm and 1.5 cm × 40 cm (values in brackets refer in the following to the smaller column); in most cases the larger column was employed unless otherwise stated. For each run, 5 ml (1.5 ml) of buffered plasma (serum in some instances) was used. The material, previously dialysed against the buffer solution, was introduced into the column and the zone allowed to move 2—3 cm below the surface of the cellulose. The electrode vessels were filled with buffer solution and the electrophoresis started. The current was 60 mA (30 mA), provided by an applied voltage of 260 V (340 V), and the duration of runs 30 h (16 h). After the completion of electrophoresis, the liquid in the column was displaced at a rate of 10 ml per hour in 2.8 ml (1.5 ml) fractions. The protein concentration of each fraction was estimated by a modified Folin procedure [5].

Assay of esterase activity. The esterase activity was determined manometrically by the Warburg technique [2] at 25°C in a bicarbonate-CO_2 buffer solution, pH 7.4, with 2.00 ml total liquid volume per flask. Results are expressed in b_{30} values, *i. e.*, the μl of CO_2 evolved during 30 min, after correction for non-enzymic hydrolysis of the substrates. The activities reported refer to initial hydrolysis rates for 0.10 ml of plasma or esterase fraction, unless otherwise stated.

RESULTS

The hydrolysis rates (values obtained with a representative sample selected from at least three animals) of various substrates by original plasma samples

separated esterase fractions using various esters as substrates. The enzyme preparation was incubated for 50 min with the inhibitor prior to addition of substrate. Code names are explained in the text.

3318CT			62C47			Atoxyl			Quinine sulphate		
A	B	C	A	B	C	A	B	C	A	B	C
<3	—	≈5	<3	—	4.8	<3	—	5.8	<3	—	5.0
		5.2			4.1			5.0			4.5
<3.5	<3	5.4	<3.5	<3.5	4.2	<3.5	<3.5	4.5	<3.5	<3.5	4.5
<3.5	<3.5	≈7	<3	<3	<5.5	<3.5	<3.5		<3		
<3	—	5.4	<3	—	4.2		—	4.1		—	4.1
<3		7.3	<3		5.9			<4			<3.5
<3		4.0	<3		3.4	<3.5		3.3	<4		4.5
<3	<3	5.2	<3	<3.5	4.7	<3.5	≈7	4.0	<3.5	<3.5	3.7
<3		4.8	<3		3.5	<3.5		<3.5	<4		4.0
<3	<3.5	4.5	<3	<3.5	3.9	<3	<3	4.3	<3	<3	<4
<3	<3	5.5	<3	<3	5.7	3.0	5.1	<2.5	4.0	<4	<3
<3.5	<3	4.4	<3	<3	4.1	<3	<3	<4	<4	<3.5	4.1

*** Values for C-esterase refer to the main peak fraction of the cholinesterase region after electrophoresis (Fig. 9).

**** Values for B-esterase refer to peak B1 (Fig. 10).

are summarized in Table 1. Activity values (b_{30} per 0.1 ml plasma) obtained with additional substrates are given in the text. The electrophoretic patterns presented in Figs. 1—12 show the rate of elution of the esterases in relation to various protein constituents, which are designated by the usual nomenclature [6]. In the legends to these figures are summarized in tabular form the relative substrate specificity and sensitivity to certain inhibitors of each esterase component. The peaks of esterase activity are designated A, B and C, corresponding to the three groups of esterases referred to in the introduction. In Table 2 the inhibitor studies are then summarized.

Human plasma (Fig. 1). The activity of human plasma in the hydrolysis of various esters has been extensively studied by many workers, and the results presented in Table 1 generally confirm previous observations, *e.g.*, that human plasma hydrolyses butyrylcholine at the highest rate of the choline esters, that the butyric acid esters of aliphatic alcohols are hydrolysed more rapidly than their other acyl homologues, and that phenyl butyrate is hydrolysed more slowly than the acetate and propionate.

On electrophoresis of human plasma two regions of high esterase activity appeared. The two peaks (A and C) are inferred to be due to the presence of at least two distinct enzymes of differing electrophoretic mobility: one (A) moved together with the albumin components and the other (C) was found between the α_2- and β-globulin fractions. A third esterase peak, demonstrated in the α-globulin region (between the peaks A and C) in most mammalian plasmata, was not detected in human plasma.

The A-esterase(s) hydrolysed phenyl acetate at a rate which was approximately 100 times greater than for phenyl butyrate. Neither the aliphatic

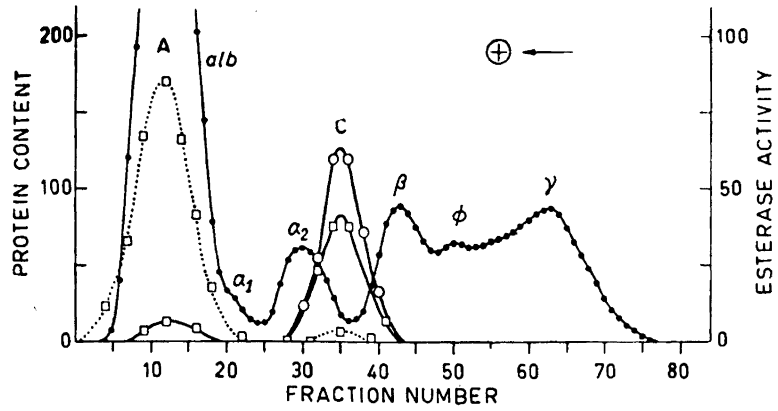

Fig. 1. Human plasma. Distribution of esterase activity and total protein after column electrophoresis (veronal buffer, pH 8.4; I = 0.1; 260 V; 60 mA; 30 h). The protein content is expressed as relative amount based on extinction values. Esterase activity is expressed in b_{30} values with the ml aliquots from each fraction as indicated below: ●——●, protein concentration; □.....□, PhAc (0.015); □——□, PhBu (0.10) and TBu (0.40); ○——○, BuCh (0.10).
Esterase activities (b_{30}/0.10 ml) against various substrates and esterase inhibition (pI_{50}) by physostigmine (Phys) and an organophosphorus compound of the peak fraction from each esterase component. Hydrolysis rates for indoxyl acetate expressed as relative amount (based on extinction values) of indigo blue formed after 30 min incubation with the peak fraction.

Esterase component	PhAc	PhBu	TBu	BuCh	BzCh	Indoxyl acetate	pI_{50}	
							Phys	Mipafox
A	560	7	0	0	0	8	<3	<3
C	24	40	10	64	7	80	7.7	6.7

nor the choline esters tested were hydrolysed by this enzyme which was resistant to all the inhibitors studied. Whether more than one arylesterase was involved in this esterase peak has not yet been established. It is possible that some human plasmata contain in low concentration an esterase running electrophoretically close to but more rapidly than the main peak fraction since the esterase peak was not uniform.

The C-esterase was a typical butyrylcholinesterase of low specificity. It also hydrolysed benzoylcholine, succinylcholine, and the butyrates of both phenol and aliphatic alcohols including glycerol. In addition, this enzyme hydrolysed procaine (b_{30} 0.5), atropine (b_{30} 0.3), Tween 20 (b_{30} 16) and the carbon analogue of acetylcholine (b_{30} 20). Cocaine and heroin hydrolyses were not detected with human plasma. The C-esterase was sensitive to all inhibitors studied (Table 2), with physostigmine being the most potent, and 62C47 and quinine the less potent compounds.

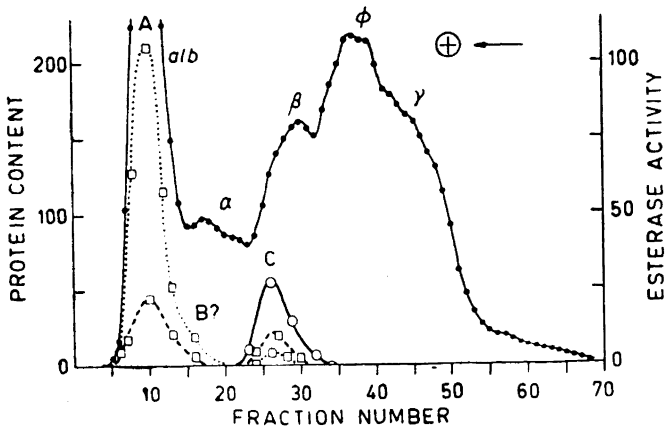

Fig. 2. Macaque-monkey plasma. Conditions as for Fig. 1. □.....□, PhAc (0.10); □— — — —□, PhPr (0.20); O———O, BuCh (0.20).

Species	Esterase component	PhAc	PhPr	PhBu	TBu	BuCh	pI_{50}	
							Phys	Mipafox
Macaque	A	105	11	1	0	0	<3	<3
	C	4	6	8	2—3	14	8.5	8.0
Mangabey	A or B	3	—	7	0	0	<3	>5
	C	50	—	95	25	150	8.4	8.0

Human plasma therefore contains two esterases. The arylesterase was responsible for 96 % of the hydrolysis of phenyl acetate and 14.5 % of that of phenylbutyrate, with the cholinesterase accounting for the remainder of the hydrolysis of these phenyl esters and for the entire activity against choline esters and triglycerides. In addition, preliminary experiments have shown that cholinesterase is responsible for more than 90 % of the hydrolysis of indoxylacetate by human plasma.

Monkey plasma (Fig. 2). Plasmata from the macaque monkey (*Macacus cynomolgus*) and the mangabey monkey (*Cercocebus torquatus*) were studied. The macaque plasma was similar to human plasma in pattern of esters hydrolysed, but the hydrolysis rates were much lower for the monkey plasma. The mangabey plasma hydrolysed choline esters at 10 times the rate of the macaque plasma, but hydrolysed the phenyl esters at a lower rate than either human or macaque plasma. Phenyl butyrate was hydrolysed more rapidly than the acetate.

The electrophoretic pattern of the macaque plasma is illustrated in Fig. 2; the legend of this figure also gives the results with the mangabey plasma. Similar to human plasma, the macaque plasma contained two distinct esterases. The A-esterase was eluted coincident with the albumin fraction and the

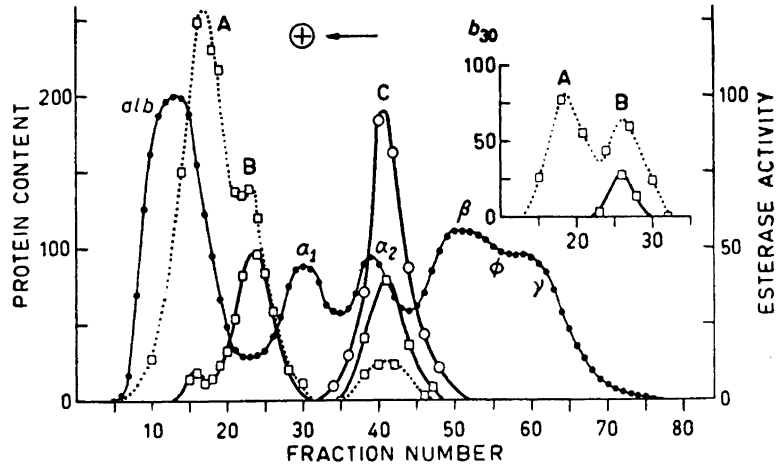

Fig. 3. Horse plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.05); □——□, PhBu (0.10); O——O, BuCh (0.10).

Esterase component	PhAc	PhBu	TPr	TBu	AmBu	PrCh	BuCh	BzCh	Indoxyl acetate	pI_{50}	
										Phys	Mipafox
A	260	9	0	0	0	0	0	0	2	<3	<3
B	140	49	41	20	18	0	0	0	20	<3	5.7
C	28	40	6	7	6	65	95	10	26	8.0	7.5

The insert is the electrophoretic pattern of a mixture of 3.8 ml of peak fraction A and 8.0 ml of peak fraction B (main diagram) showing clearer resolution of the enzymes. □ ..... □, PhAc (0.40); □——□, PhBu (0.40).

C-esterase between the α_2- and β-globulins. The first enzyme was a typical acetylarylesterase which was resistant to both physostigmine and certain organophosphorus compounds. Although aliesterase (B) was not detected in the macaque plasma, the activity may have been too low after electrophoresis to be determined by the techniques employed.

The butyrylcholinesterase (C) of the macaque plasma was present in low concentration compared with human and mangabey plasmata. This esterase was also responsible for the hydrolysis of tributyrin and for up to 65 % of the plasma activity against phenyl butyrate.

The mangabey plasma probably contained a B-esterase in comparatively low concentration. The low activity of the fractions did not allow assay of this enzyme for inhibition by organophosphorus compounds. Since phenyl butyrate was hydrolysed more rapidly than phenyl acetate and the esterase was eluted later than the A-esterase of macaque plasma, it appears likely that the mangabey esterase was a B-rather than an A-estrase. The butyrylcho-

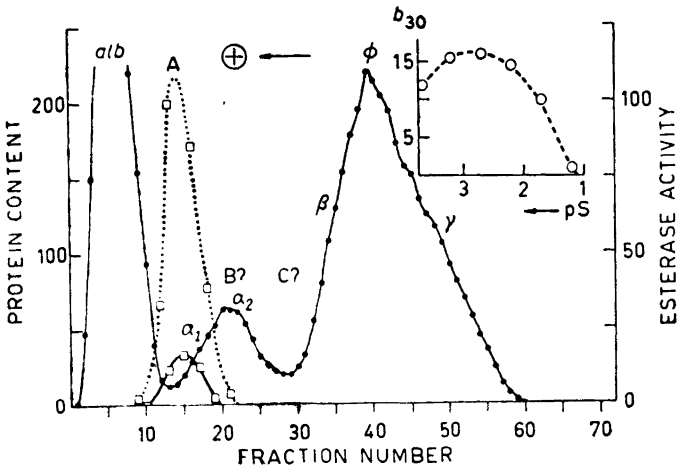

Fig. 4. Cow plasma. Results obtained with a sample from calf. Conditions as for Fig. 1. □.....□, PhAc (0.01); □——□, PhBu (0.40).

Esterase component	PhAc	PhBu	TBu	PrCh	pI_{50}	
					Phys	*iso*-OMPA
A	2 180	10	0	0	<3	<3
B	?	?	<1	0	<3	≈6
C	?	?	<1	<1	7.3	≈6

The insert is the activity-pS curve for the hydrolysis of propionylcholine by cow plasma (0.40 ml).

linesterase, present in high concentration, was responsible for the hydrolysis of tributyrin.

Horse plasma (Fig. 3). The pattern for hydrolysis of various esters by horse plasma was similar to that for human plasma except for the hydrolysis of the triglycerides, where tripropionin was optimal for horse plasma and tributyrin for human plasma.

Horse plasma contained three types of esterases resolved in three distinct peaks by electrophoresis. The A- and B-esterases migrated between the albumin and α_1-globulin components, and the C-esterase between the α_2- and β-globulins. The A-esterase was a typical acetylarylesterase, being resistant to all inhibitors studied. *p*-Nitrophenyl acetate was hydrolysed at a lower rate than phenyl acetate. The B-type esterase hydrolysed both aromatic and aliphatic esters, the propionates being split at a higher rate than the corresponding acetates and butyrates. This enzyme was also responsible for the hydrolysis of "Tween 20" (b_{30} 35). It was less sensitive to organophosphorus compounds than most B-esterases; *e.g.*, this esterase was resistant to *iso*-OMPA, while mipafox gave the same relatively low pI_{50} value (5.7), when this

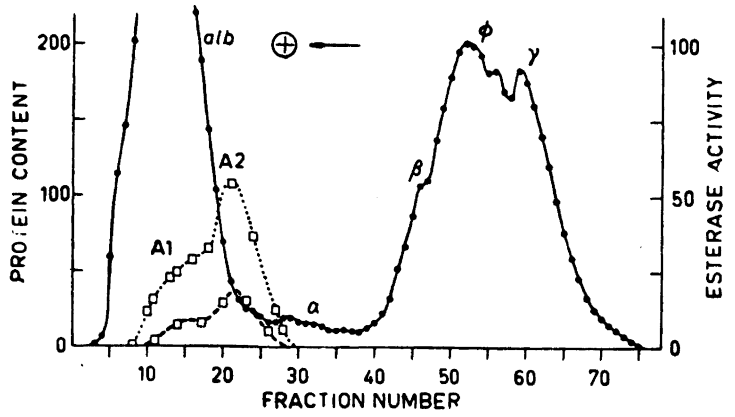

Fig. 5. Reindeer plasma. Conditions as for Fig. 1. □.....□, PhAc (0.40); □— — —□, PhPr (0.40). N.B.: the activity of the arylesterases was much lower than expected after electrophoresis (*cf.* Table 1).

Esterase component	PhAc	PhPr	PhBu	PrCh	pI_{50}	
					Phys	*iso*-OMPA
A1	7	2.5	?	0	<3	<3
A2	14	5	<1	0	<3	<3
C	?	?	<1	<1	7.2	7.6

B-esterase was assayed with a number of various substrates. Poor inhibitor efficiency was also observed with the quaternary ammonium compounds, the phenothiazine derivative (Astra 1397), atoxyl, and quinine sulphate. A better separation of the A-and B-esterases was accomplished by re-electrophoresis of the two peak fractions (inserted diagram of Fig. 3).

The C-esterase was a typical butyrylcholinesterase having the lowest specificity of the three esterases present. Its inhibition pattern was similar to that of human plasma cholinesterase. At least three enzymes in horse plasma hydrolyse aromatic esters since the cholinesterase was also active on these substrates. Indoxyl acetate was also hydrolysed by all three esterases, the C- and B-esterases being more active than the arylesterase.

Cow plasma (Fig. 4). Of the substrates tested, only the aromatic esters were hydrolysed at appreciable rates. The enzyme catalysing this reaction was a typical acetylarylesterase which had a lower electrophoretic mobility relative to that of the albumin components than was usually found for other mammalian plasma arylesterases.

Inhibitor experiments with the original cow plasma showed that tributyrin was hydrolysed by two esterases both present in very low concentration. Cholinesterase accounted for 80 % of the total hydrolysis of this ester with the remainder being hydrolysed by an esterase (probably a B-type esterase)

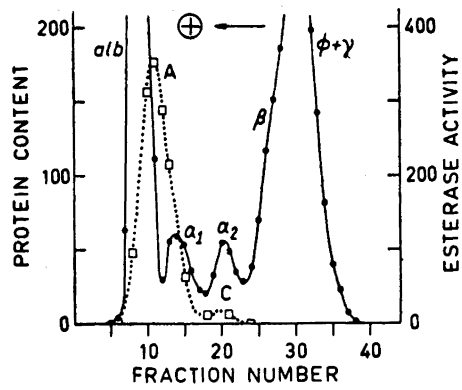

Fig. 6. Goat plasma. Electrophoresis was carried out on a small column. Otherwise, conditions were as for Fig. 1. □ ..... □, PhAc (0.10).

Esterase component	PhAc	AcCh	pI_{50}		
			Phys	Mipafox	3318 CT
A	360	0	<3	<3	<3
C	15	1	7.5	<4	7.3

resistant to physostigmine and sensitive to mipafox and *iso*-OMPA. The activities of the B- and C-esterases were too low to be detected in fractions after electrophoresis.

The proportion between the hydrolysis rates for phenyl acetate and phenyl butyrate was 47:1 for the whole plasma and 218:1 for the arylesterase peak fraction; thus the activity against the butyrate was reduced much more than that against the acetate by dialysis and electrophoresis. It appears therefore that the phenyl esters were hydrolysed by at least two enzymes of cow plasma. The sensitivity of these esterases to organophosphorus compounds was not successfully tested.

The physostigmine-sensitive esterase was a propionylcholinesterase which was inhibited by excess propionylcholine (see inserted graph of Fig. 4). This esterase was sensitive to those compounds (3318CT and 62C47) which are regarded as being selective inhibitors of acetylcholinesterase (of erythrocytes and nerve tissue), and was resistant to "Astra 1397". The cholinesterase of cow plasma therefore differs greatly from the cholinesterase present in most other mammalian plasmata.

Reindeer plasma (Fig. 5). As noted for other ruminant plasmata, the reindeer plasma hydrolysed most esters at a very low rate. Although the aromatic esters were attacked more readily than the other substrates even here the hydrolysis rates were comparatively low. Two esterase peaks were found after electrophoretic separation; they were due to the presence of two acetylarylesterases (A1 and A2), the properties of which were similar, in that both

enzymes were resistant to *iso*-OMPA and physostigmine. This plasma probably does not contain any B-type esterase.

The cholinesterase present in low concentration was a butyryl- (or propionyl-) cholinesterase, the activity of which was not inhibited by high substrate concentration. This enzyme was responsible for 20 % of the plasma activity against phenyl butyrate.

Goat plasma (Fig. 6) *and sheep plasma.* The goat plasma hydrolysed phenyl acetate at a very high rate due to the presence of an acetylarylesterase. A few per cent of the phenyl acetate hydrolysis was due to a second esterase. Inhibition experiments showed this second enzyme to be a cholinesterase which had the properties of an acetylcholinesterase. It was inhibited by high concentration of acetylcholine which explains why the peak fraction C hydrolysed phenyl acetate at a higher rate than acetylcholine with the substrate concentration used (1.0×10^{-2} M). The quaternary ammonium compounds were comparatively strong inhibitors and the phenothiazine derivative (Astra 1397) was not active as an inhibitor of the acetylcholine hydrolysis. Moreover, this enzyme was only sensitive to comparatively high concentration of the organophosphorus compounds, atoxyl and quinine (Fig. 6 and Table 2).

Sheep plasma showed much the same esterase pattern as goat plasma, both regarding hydrolysis rates of various esters (Table 1), observed esterase peaks of the electrophorogram, and esterase sensitivity to various inhibitors.

Swine plasma (Fig. 7). The arylesterase concentration of swine plasma exhibits considerable individual variation (b_{30} 20—900). The results obtained with a sample having a b_{30} of 450 will be considered here. More details on swine plasma esterases, including evidence for the inheritance of the plasma arylesterase have been reported in a previous paper [7].

The main esterase present in the plasma sample analysed was an acetylarylesterase. *Para*-substitution of phenyl acetate with a nitro group increased the enzymic hydrolysis rate about eight times. With other plasma arylesterases of mammals such a substitution in the aromatic ring usually greatly decreases the hydrolysis rate. The swine esterase was resistant to all inhibitors tested. By contrast, the hydrolysis of phenyl acetate by a plasma exhibiting low activity against this substrate was strongly inhibited by all those compounds which were strong inhibitors of the cholinesterase present in low concentration. This suggests that the hydrolysis of phenyl acetate by this type of plasma was due mainly to the plasma cholinesterase. The less active the plasma was in hydrolysing phenyl acetate, the more closely did the pattern of inhibition, when assayed with phenyl acetate, resemble that of cholinesterase.

The arylesterase migrated on electrophoresis together with the albumin components. The activities against phenyl acetate and its *p*-nitro derivative moved coincident with each other. No B-esterase or other arylesterases were detected.

Due to the low activity of swine plasma on choline esters, a cholinesterase could not be traced accurately in the fractions obtained in these electrophoresis experiments. However, by running the electrophoresis on smaller columns it was possible to find this type of esterase between the α_2- and β-globulins. Experiments with whole plasma revealed that this esterase was a butyryl-

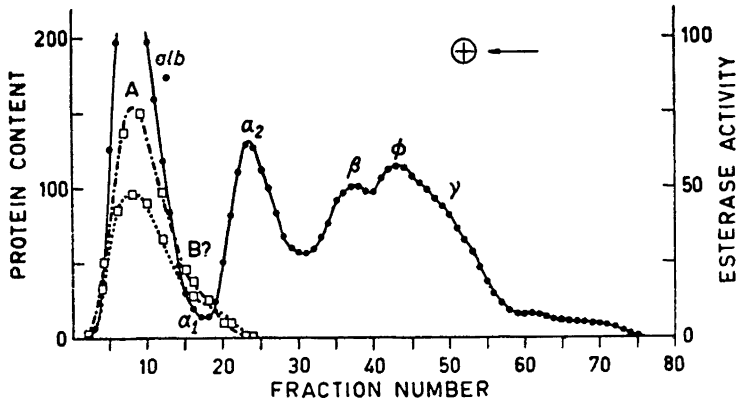

Fig. 7. Swine plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.10); □ —.—.□, *p*-nitrophenyl acetate (0.02).

Esterase component	PhAc	*p*-Nitro-PhAc	BuCh	pI_{50}	
				Phys	Mipafox
A	98	775	0	<3	<3
B?	?	?	0	<3	?
C	<1	?	<1	7.0	6.5

cholinesterase, the specificity of which differed from those of all other vertebrate plasmata by hydrolysing acetylcholine more rapidly than propionylcholine confirming previous results [8,9]. Benzoylcholine and succinylcholine were also hydrolysed by this enzyme. The hydrolysis of succinylcholine was unusual in that the swine plasma hydrolysed it more rapidly (comparable protein concentrations) that any other animal tissue so far studied. The hydrolysis rate of the carbon analogue of acetylcholine (3,3-dimethylbutyl acetate) was 10 % of the hydrolysis rate of the choline ester. Acetyl- β-methylcholine was not split. The swine cholinesterase also hydrolysed phenyl acetate, the hydrolysis rate being approximately the same as for butyrylcholine. In newborn animals, which irrespective of the plasma activities of the parents had low arylesterase activity, more than 97 % of the hydrolysis rate of phenyl acetate was due to a butyrylcholinesterase.

The inhibition experiments performed with swine plasma esterases have been previously reported [7].

Cat plasma (Fig. 8). The high hydrolysis rate for aromatic esters by this plasma was due to an arylesterase (probably an acetylarylesterase, in contrast to previous statements [1,10]) and a B-type esterase. The latter enzyme hydrolysed a variety of esters, the hydrolysis rates being greatly dependent on the configuration of the alcoholic group. Tripropionin was hydrolysed at a higher rate than tributyrin, the reverse being true for the phenyl and *iso*amyl derivatives. The most characteristic property of this enzyme was its high sensitivity

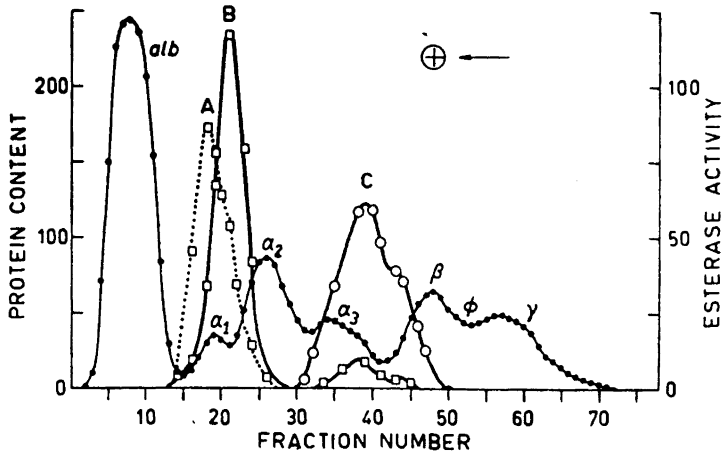

Fig. 8. Cat plasma. Conditions as for Fig. 1. □ . . . . . □, PhAc (0.01); □———□, PhBu (0.10); ○———○, BuCh (0.40), PrCh (0.80).

Esterase component	PhAc	PhBu	TPr	PrCh	BuCh	pI_{50}	
						Phys	*iso*-OMPA
A	870	≈10	0	0	0	<3	<3
B	<100	117	54	0	0	≈3.5	≈7
C	≈5	10	0	8	16	7.75	7.2

to atoxyl (pI_{50} 7), not found with any other plasma esterases studied in this series. Due to the unusually high mobility of the albumin component [6], the A-esterase fractions were all free from these proteins.

The cholinesterase of cat plasma was similar to other mammalian butyrylcholinesterases. It was responsible for approximately 10 % of the plasma activity against phenyl butyrate and for less than 1 % of that against phenyl acetate. The activity was found after electrophoresis between the α_3- and β-globulins and was not distributed in an uniform esterase peak. The enzyme protein seemed to move partly together with other protein constituents (possibly by complex formation). No evidence could be found by inhibition experiments and specificity studies with various fractions forming the peak or peaks that more than one cholinesterase was present. The same observations were made with three various plasma samples subjected to electrophoretic separation.

Dog plasma (Fig. 9). The esterase pattern of this plasma resembled that of human plasma in the hydrolysis of choline and aromatic esters. In comparison with human plasma the aliphatic esters were hydrolysed at a comparatively low rate by dog plasma. The main esterases present were an acetylarylesterase and a butyrylcholineasterase. Beside the main arylesterase

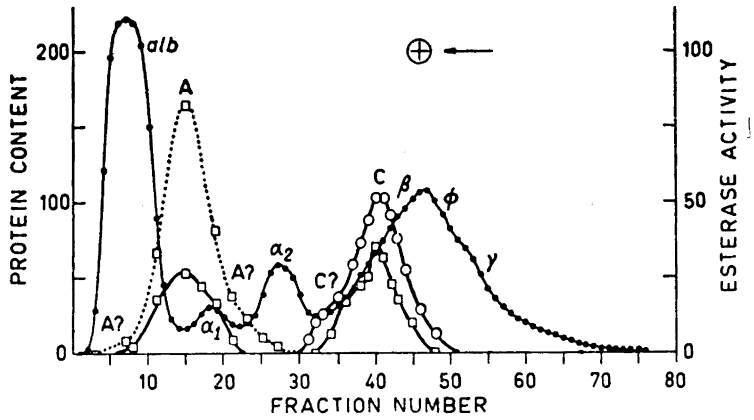

Fig. 9. Dog plasma. Conditions as for Fig. 1. □ . . . . . □, PhAc (0.025); □——□ PhBu (0.20); O——O, BuCh (0.20).

Esterase component	PhAc	PhBu	TPr	PrCh	BuCh	pI_{50}	
						Phys	*iso*-OMPA
A	248	14	0	0	0	<3	<3
C	<1	18	2	17	25	8.5	7.7

peak. fractions migrating on electrophoresis at a higher rate (together with the albumins) and at a lower rate (close to the α_2-globulins) were active in hydrolysing phenyl acetate. The esterases of both these "extra" fractions were resistant to *iso*-OMPA. Whether these activities were due to separate enzymes or merely due to the presence of traces of the arylesterase has not been further investigated.

The butyrylcholinesterase was found between the α_2- and β-globulins. The esterase peak was not uniform. Part of the enzyme(s) (C?) possibly moved together with other protein constituents. The specificity and sensitivity to inhibitors were the same for the active esterase constituents present in the main peak fraction and in the more rapid-moving fractions. In the cholinesterase region a B-type esterase might well have been present because the enzymic hydrolysis of phenyl butyrate by some fractions (B?) was inhibited by physostigmine only when this agent was present in higher concentration (10^{-4} M) than was necessary to inhibit completely the hydrolysis of a choline ester. If was found that 20—30 % of the phenyl butyrate hydrolysis by dog plasma was due to the cholinesterase which displayed the maximum activity against tripropionin. Before the complexity of the esterase fractions between the α_2- and β-globulins has been further investigated, it cannot be stated whether dog plasma contains a B-type esterases and more than one cholinesterase.

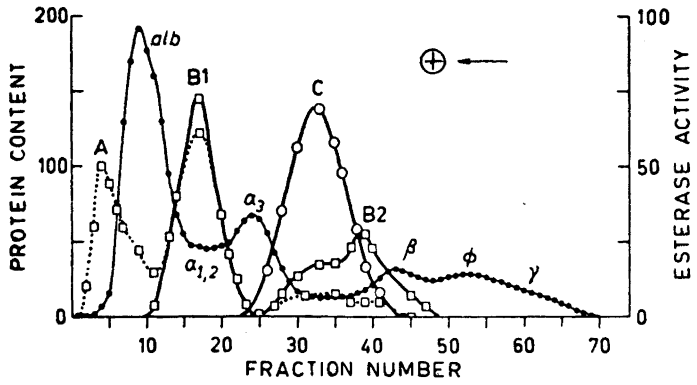

Fig. 10. Guinea-pig plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.05); □———□, PhBu (0.05); O———O, BuCh (0.20).

Esterase component	PhAc	PhBu	AmPr	AmBu	TPr	TBu	Tween	BuCh	pI_{50}	
									Phys	*iso*-OMPA
A	100	<1	0	0	0	0	0	0	<3	<3
B1	120	145	15	3	110	108	22	0	<3	5.0
B2	10	55	10	2	8	8	0	0	<3	5.9
C	15	35	0	0	10	12	0	35	8.5	7.0

Guinea-pig plasma (Fig. 10). In the series of aromatic and aliphatic esters tested the propionates were hydrolysed at the highest rate. Phenyl acetate and propionate were split by at least four enzymes. The first esterase fraction eluted following electrophoresis was a propionylarylesterase which migrated more rapidly than the albumins and was responsible for about 40 % of the total hydrolysis of phenyl acetate; the assay method was not sensitive enough to measure the hydrolysis rate of phenyl butyrate in these fractions. Two esterases resistant to physostigmine but sensitive to organophosphorus compounds were separated. One (Bl) was found in the α-globulin region and hydrolysed the propionates and butyrates at a higher rate than the acetates of the aliphatic and aromatic esters. This esterase was responsible for 50 % of the hydrolysis of phenyl acetate and 60—70 % of that of phenyl butyrate. The hydrolysis of ethyl acetate and ethyl butyrate as well as of "Tween 20", the rate of which was comparatively high (b_{30} 116) with this plasma, was also catalysed by this enzyme. The second B-type esterase (B2) migrated on electrophoresis at a higher rate than cholinesterase. The *iso*amyl esters were hydrolysed by both the B-esterases but not by the C-type esterase, which had the properties of a butyrylcholinesterase. In addition to the hydrolysis of choline esters, including benzoylcholine, this C-esterase was responsible for about 15 % of the plasma activity against phenyl butyrate and 5—10 % of that against phenyl acetate.

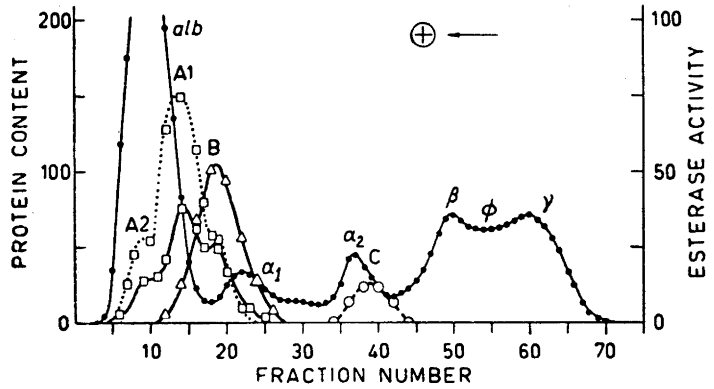

Fig. 11. Rabbit plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.02); □——□ PhBu (0.10); △——△, TBu (0.40); ○——○, PrCh (0.40).

Esterase component	PhAc	PhBu	TBu	PrCh	Indoxyl acetate	pI_{50}		
						Phys	*iso*-OMPA	62C47
Al	375	40	?	0	20	<3	<3	<3
A2	135	15	0	0	?	<3	<3	<3
B	?	25	12	0	45	<3	6.9	<3
C	<1	<1	<1	4	30	6.4	<4	5.7

Guinea-pig plasma was highly active in hydrolysing the carbon analogue of acetylcholine (b_{30} 150). It was characteristic of the reaction that the velocity fell off greatly with time. The enzyme responsible was the physostigmine resistant B-type esterase (B1). Heroin was also hydrolysed at a comparatively high rate by this plasma (b_{30} 12).

Rabbit plasma (Fig. 11). The esterase activity of rabbit plasma varied considerably from animal to animal, especially when aromatic esters were used as substrates. The activity of the enzymes responsible for the hydrolysis of these esters were reduced in varying degrees by dialysis against the veronal buffer. The electrophoretic pattern was in most instances difficult to interprete. There were at least three enzymes in the albumin and α_1-globulin regions which hydrolysed aromatic esters. The main esterase peak (A1) was due to an acetylarylesterase. Active fractions (A2) moving together with the albumins had esterase properties similar to those of the fractions of the main esterase peak (A1), but whether the two peaks were due to the same or different enzymes has not been further investigated.

The B-type esterase hydrolysed aliphatic and aromatic propionates and butyrates at higher rates than the acetates. It was much more sensitive to the organophosphorus compounds and to atoxyl than was the C-type esterase of this plasma. Great individual variation occurred in the concentration of this esterase.

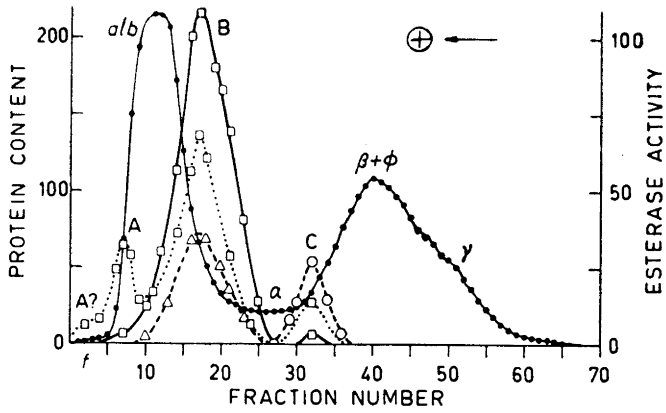

Fig. 12. Rat plasma. Conditions as for Fig. 1. □ . . . . . □, PhAc (0.05); □———□, PhBu (0.05); △— — —△, TPr (0.10); ○— — —○, PrCh (0.40).

Esterase component	PhAc	PhBu	AmAc	AmBu	TPr	Tween	PrCh	BuCh	pI_{50}	
									Phys	*iso*-OMPA
A	60	6	0	0	0	0	0	0	<3	<3
B	140	220	11	20	35	13	0	0	<3	5.8
C	4	1	0	0	0	0	7	4	6.3	6.7

The cholinesterase present in comparatively low concentration has the properties of an acetylcholinesterase. The activity was inhibited by high concentrations of acetylcholine (and propionylcholine). The enzyme was resistant to "Astra 1397", atoxyl and quinine, and was less sensitive to *iso*-OMPA and mipafox than other plasma cholinesterases. On the other hand, the quaternary ammonium compounds were comparatively strong inhibitors. These observations indicate that the rabbit plasma cholinesterase is of a type characteristic of acetylcholinesterase present in erythrocytes and nervous tissue of various animal species.

Preliminary experiments showed that indoxyl acetate was hydrolysed by all three types of esterases present in rabbit plasma, with the B- and C-esterases being the most active ones. This plasma hydrolysed heroin (b_{30} 10) and cocaine (b_{30} 5) at a higher rate than most other mammalian plasmata.

Rat plasma. (Fig. 12). The acetylarylesterase moved in electrophoresis at a higher rate than the albumins. Its activity was reduced during dialysis against the veronal buffer to a much greater degree than with the other esterases of this plasma. In the small amount of protein (f-component), moving faster than the albumins, arylesterase was also present. The B-type esterase hydrolysed phenyl butyrate and *iso*amyl butyrate at a higher rate than the corresponding acetates and propionates, and tripropionin more rapidly than its other acyl homologues. The broad B-esterase peak obtained

after electrophoresis probably consisted of a single enzyme because the activities of all fractions therein showed the same sensitivity to *iso*-OMPA when various esters were used as substrates. This esterase together with cholinesterase was responsible for about 40 % of the plasma hydrolysis of phenyl acetate.

The rat was one of the few mammalian species studied in this series (cf. cow, above) which had a typical propionylcholinesterase in its plasma, confirming previous observations [8]. A similar enzyme was present in mouse plasma. The propionylcholinesterase of rat plasma differed from the butyrylcholinesterases present in other plasmata in respect to inhibitory effects of various compounds (Table 2) and hydrolysis rates of choline and non-choline esters; *e.g.*, acetyl-β-methylcholine and benzoylcholine as well as phenyl acetate and propionate were split at relatively high rates.

Arylesterases as thiol enzymes. All the arylesterases studied were resistant to the selective inhibitors used (Table 2). Introductory studies were therefore performed to find inhibitors of these enzymes. Of the three main types of agents reacting with thiol groups, *p*-hydroxymercuribenzoate, iodoacetamide and *o*-iodosobenzoate, the first compound was the only powerful inhibiting agent of four different plasma arylesterases (human, cow, swine, rabbit) (pI_{50} 5—6). This suggests the presence of an active SH-group at the enzyme surface of these esterases. Aliesterases and cholinesterases were resistant to the mercaptide-forming agent (*p*-hydroxymercuribenzoate) and the alkylating agent (iodoacetomide). The oxidizing agent (*o*-iodosobenzoate) was a comparatively strong inhibitor (pI_{50} 5.0) of the cholinesterases of human and horse plasmata. A full account of these studies will be reported later.

DISCUSSION

The nature of plasma esterases has been the subject of investigation and speculation for nearly forty years. These enzymes have usually been characterized based on their substrates and sensitivity to inhibitors. Differentiation was based mainly on work with original plasma (serum) or crude enzyme preparations. Homogeneous esterase preparations such as plasma cholinesterases free from other esterases, were only rarely employed. A classification of plasma esterases is difficult to deduce from such studies due to much overlapping of properties and species differences.

As a preliminary to the present study, various preparative methods were tested for separating the esterases of vertebrate plasmata. Fractionation with ammonium sulphate gave poor separation of the two esterases present in human plasma, and even less promising results with plasmata where a more complex esterase pattern was involved. Ethanol fractionation at low temperature resulted in good separation of the two human plasma esterases. This technique was therefore employed with a number of other plasmata *, but was inadequate for separating the aryl- and aliesterases.

Except for a few studies of plasma cholinesterases [11,12] and of A- and B-esterases [13], no attempt has been made so far to separate electrophoretically

* This investigation was carried out in collaboration with Ing. H. Nihlén of Forskningslaboratoriet LKB, Stockholm.

plasma esterases. Preparative electrophoresis on cellulose columns was choosen as the technique for the present investigation, because of its adaptability for the isolation of plasma proteins in sufficient amounts to enable a study of their properties [14,15]. Phosphate and borate were found to be unsuitable as buffers for electrophoresis since the arylesterases and the aliesterases in many plasmata were not separated in these buffers. The best separation of plasma esterases was obtained with veronal buffer (pH 8.4 and $I = 0.1$). However, the various types of esterases were influenced differently by dialysis against this buffer. Species as well as esterase differences were noted in this respect. For this reason the activities of separated enzymes are not always comparable with those calculated from activity values obtained with original plasma.

The esterase peaks after electrophoresis are shown in graphs giving their relative position to known protein constituents. The absolute electrophoretic mobilities of the proteins from various plasmata cannot be compared as the fraction numbers plotted as abscissae are not the same for all plasmata. However, all electrophoresis runs and elutions were carried out under identical conditions, with the exception of temperature which varied from 5°C to 11°C in various runs.

Three types of esterases were shown for mammalian plasmata, based on electrophoretic mobility relative to known protein constituents, substrate specificity, and susceptibility to selective inhibitors. Some plasmata contain all three types, others two and still others only one. Each group of enzymes has several types, and every animal species has its own typical set of plasma esterases. When designating these esterases, the nomenclature generally accepted by most authors in this field was followed as far as possible. Since the three types of esterases have different electrophoretic mobilities under the experimental conditions used, they could be conveniently studied when separated after electrophoresis. Cholinesterases had in most cases much lower electrophoretic mobility than the aryl- and ali-esterases and were therefore easily obtained free from other esterases. Aryl- and aliesterases migrated more closely, but in most cases they could be studied separately and not in mixtures.

The presence of an aromatic esterase in human plasma has been proposed by Mounter and Whittaker [16]. Aldridge [10] found two types of serum esterases of several species hydrolysing *p*-nitrophenyl acetate, propionate and butyrate. The enzyme not inhibited by paraoxon (diethyl *p*-nitrophenyl phospate) and hydrolysing acetate at a higher rate than butyrate was called A-esterase, and the sensitive enzyme B-esterase. It turned out in the present investigation that the A-esterase is the enzyme which has the greatest electrophoretic mobility, and is resistant not only to paraoxon but to a number of other organophosphorus compounds and to physostigmine. Since this esterase hydrolyses various aromatic esters (in addition to phenyl esters, the acetates of α- and β-naphthol and indoxyl) but not aliphatic and choline esters, it was called *arylesterase*. Whether this type of esterase is also responsible for the hydrolysis of certain organophosphorus compounds will be the subject of a subsequent study. In contrast to other plasma esterases the arylesterases are highly sensitive to *p*-hydroxymercuribenzoate which suggests the presence of an active SH-group at the enzyme surface. All mammalian plasmata studied

contain this type of esterase in high concentration and it is the predominant esterase present in the plasmata of ruminants and swine.

In the earliest investigation on plasma esterases such simple esters as alkyl butyrates, fats and oils were used as substrates. "Simple esterases" having a substrate preference for short-chained fatty acid esters of simple alcohols were differentiated from "lipases" which were more active in hydrolysing esters of glycerol and fatty acids with long chains. Later it was observed that choline esters were also hydrolysed by the plasmata of several species, and that the enzyme responsible was different from "esterase" and "lipase". This led to a large amount of work on substrate specificity of plasma esterases. No clearcut line of differentiation between the various enzymes could be drawn, until Richter and Croft [17] made the observation that all enzymes classified as "cholinesterases" were completely inhibited by 10^{-5} M physostigmine, while "simple esterases" were resistant to this inhibitor dosage. These authors introduced the term *aliesterase* for the latter type of esterases. No evidence has been given so far as to the identity of this esterase type with the A- or B-esterases. It was demonstrated in the present investigation that the esterase migrating electrophoretically next to the arylesterase has the properties of a B-esterase and an aliesterase, being highly sensitive to certain organophosphorus compounds and resistant to 10^{-5} M physostigmine. This esterase hydrolyses both aliphatic and aromatic esters. Although the term aliesterase is not adequeate for this type of esterase, since some of them hydrolyse aromatic esters at a higher rate than the corresponding aliphatic ones, its use is recommended because the definition introduced by Richter and Croft is still valid. Triglycerides are also hydrolysed by this esterase, and since no special esterase hydrolysing only these esters has been detected in plasma, lipases are included in the aliesterases. Human, monkey, dog, swine and ruminant plasmata do not contain aliesterase. The hydrolysis of aliphatic esters by these plasmata is catalysed only by cholinesterases, which in these cases have a particularly low specificity for choline esters. The mammalian aliesterases of the plasmata of horse, cat, guinea-pig, rabbit and rat hydrolyse the butyrates and propionates at a higher rate than the acetates. Acetylaliesterases, however, exist in the plasmata of some lower vertebrates, as will be discussed more fully in the second paper of this series.

The specificity of *cholinesterases* present in the plasmata of various mammalian species has been studied extensively in previous investigations [8, 18, 19]. By ammonium sulphate fractionation the human plasma cholinesterase is precipitated with the albumins [18, 20, 21], but on electrophoresis it migrates between α_2- and β-globulins [11, 12]. It was demonstrated in the present investigation that this relatively low mobility under the experimental conditions used was valid also for the cholinesterases of other mammalian plasmata. These plasma esterases were therefore easily separated from the aryl- and aliesterases by electrophoresis, and for practical reasons they were designated "C-esterases" in the present study. The hydrolysis of choline esters and the high sensitivity to physostigmine are properties sufficiently distinct to regard the cholinesterases as a separate group of esterases. When only the sensitivity to certain organophosphorus compounds is considered, cholinesterases might be regarded as B-type esterases [22], but plasma cholinesterases are definitely distinct enzy-

mes differing electrophoretically and in their characteristics as esterases from the aliesterases as clearly as do the arylesterases.

Cholinesterases were present in all mammalian plasmata studied except in those of the ruminants where their activities were very low or too low for detection. Various types of plasma cholinesterases exist and overlapping of properties is very marked. This is especially true for the enzymes present in the plasmata of rabbit, swine, cow and reindeer. A butyrylcholinesterase is characteristic of several of the mammalian plasmata studied, and a propionylcholinesterase is present in rat plasma. As will be demonstrated in a second paper of this series, butyrylcholinesterase does not occur in the plasmata of lower animals, where propionyl- and acetylcholinesterases are the characteristic physostigmine-sensitive esterases instead.

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