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ON THE IMMUNOLOGICAL INTER-RELATIONSHIPS OF THE VERTEBRATE ESTERASES

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SUMMARY

1. In order to assess the immunological inter-relationships of the vertebrate esterases, antisera were produced against several purified enzymes from the different esterolytic classifications, namely sheep liver carboxylesterase (carboxylic-ester hydrolase, EC 3.1.1.1), horse serum cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8), ox testis acetylesterase (acetic-ester hydrolase, EC 3.1.1.6) and ox serum arylesterase (acyl-ester hydrolase, EC 3.1.1.2).

2. These antisera were tested for their interactions at the level of enzyme heterogeneity, by gel electrophoresis of a representative range of tissues before and after incubation with the sera, followed by observation of the alteration in the patterns of esterase heteromorphs consequent upon this treatment.

3. From these results, it appears that a close immunological relationship exists between the multiple forms of each individual esterase type within a single species. In the case of the acetylesterases, this immunological relationship extends between the species as well. A close relationship was also observed between chicken liver carboxylesterase and horse serum cholinesterase, and to a lesser degree between these two latter enzymes and sheep liver carboxylesterase.

4. In addition to the electrophoretic studies, complement fixation inhibition assays were carried out between each of the antisera and all the purified antigens. Also, micro-complement fixation assays between the antisera to the chicken liver carboxylesterase and horse serum cholinesterase were effected with all the antigens. These immunological tests with pure enzymes served to verify that a degree of relationship exists between the horse cholinesterase and the chicken carboxylesterase.

5. The significance of these results has been discussed in relation to the phylogenetic variability of the esterases, the complex structural and genetic inter-relationships of these multiple enzyme forms, and the comparative situation in intensively studied isoenzyme systems.

Abbreviation: R.M. relative mobility.

INTRODUCTION

The hydrolysis of carboxylesters has been shown to be effected by a wide variety of proteins in vertebrate tissues[1–7]. Four main types of soluble esterase activity have been identified; namely: carboxylesterases (carboxylic-ester hydrolase, EC 3.1.1.1); arylesterases (aryl-ester hydrolase, EC 3.1.1.2); acetylesterases (acetic-ester hydrolase, EC 3.1.1.6) and cholinesterases (acylcholine acyl-hydrolase, EC 3.1.1.8), and within each of these classes, an extensive and species-specific multiplicity has been reported. Because of the complex nature of this heterogeneity, the clarification of the inter-relationships of these esterolytic heteromorphs has proved to be a substantial problem, and in spite of previous investigations into the multiplicity, substrate and inhibitor characteristics, stabilities and molecular weight relationships of these enzymes, there remains a need for additional clarification[1–7,33]. Recently, some of the principle esterase types have been isolated in purified forms (refs 8–13; K. Scott, M. T. C. Runnegar and B. Zerner, personal communication; P. Inkerman and B. Zerner, personal communication), and this has enabled assessment of the complex inter-relationships of this group of proteins by an additional parameter (*viz.* an immunological comparison).

In the present study, antibodies prepared against the principle carboxylesterases of sheep (K. Scott, M. T. C. Runnegar and B. Zerner, personal communication) and chicken liver (P. Inkerman and B. Zerner, personal communication), horse serum cholinesterase[13,14], ox serum arylesterase[8], ox acetylesterase[9] and bovine serum albumin, have been used in micro-complement fixation experiments, to ascertain the nature of immunological relationships, which exist between these proteins. Also, the relationships between the purified proteins and their multiple forms in vertebrate tissues, and between the multiple forms of esterases that exist in other species, have been investigated by immunoelectrophoretic techniques.

METHODS

Carboxylesterases purified from chicken (K. Scott, M. T .C. Runnegar and B. Zerner, personal communication) and sheep livers (P. Inkerman and B. Zerner, personal communication) were the generous gift of Professor B. Zerner from this department; purified ox serum arylesterase was donated by Mr B. Kitchen[8], Department of Primary Industries, Brisbane, Australia; purified ox testis acetylesterase[9] was prepared by the authors, and purified bovine serum albumin and horse serum cholinesterase were obtained from Sigma Chemical Company. Antisera to these individual proteins were prepared in New Zealand white rabbits by injections of between 5 and 10 mg of the purified protein. When analysed by double diffusion on agar[15], these sera produced only a single precipitation band with the complementary-purified protein, and gave no precipitation bands against any other of the pure proteins.

Antisera were also tested for specificity against homogenates of tissues containing esterases, using the immunoelectrophoretic technique of Holmes and Markert[16]. Each antiserum prepared against a pure protein was preincubated with the tissue homogenates of rat, guinea pig, chicken, ox, pig, sheep and horse, which had been selected so as to contain all the multiple forms of esterases found in the individual species[1–5]; these preincubated antisera plus homogenates were electrophoresed in

parallel with untreated homogenates[16–19]. All gels were stained for esterase activity, the individual bands classified by reference to their specificity, inhibitory characteristics and relative mobility[1–7], and the effect of addition of antisera noted. This methodology is illustrated in part in Fig. 4.

Complement fixation inhibition assays were performed by a modification of the technique of Wasserman and Levine[20] and Osler *et al.*[21], developed to cope with the special requirements of the present investigation (*e.g.* the small amount of some of the purified enzymes available), and using Microtiter instruments (Cook Engineering Company, Alexandria, Va). A control experiment using the non-modified technique of Wasserman and Levine[20] was also run in order to provide ample confirmation of the results obtained with the above method.

In all cases the diluent used was isotonic veronal buffer. Commercially prepared sheep erythrocytes were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia, in sterile Alserver's solution, and stored at 4 °C. Optimum conditions for dilution of the red cells and ease of observation of haemolysis were found to be obtained with $1.1 \cdot 10^8$ erythrocytes per ml, and suspensions were standardized to this concentration by diluting the stock cells so that a 6.6% solution in 0.1% aqueous Na_2CO_3 gave an absorbance at 541 nm of 0.12. Complement, also commercially prepared by the Commonwealth Serum Laboratories, was obtained in ampoules containing lyophilized complement from 2.2 ml of guinea pig serum. It was found that in this system with the above dilution of red cells and the conditions to be stated, a 1 in 100 dilution of reconstituted complement gave 50% haemolysis. Bacto-antisheep haemolysin (Difco Laboratories, Detroit, Mich., U.S.A.), was used to sensitize the erythrocytes. Estimation of the dilution of haemolytic antibody was made, and it was found that at dilutions of 1/100 of complement, a constant 50% haemolysis was obtained using a constant dilution of 1/6400 of haemolysin.

Titration was carried out on plates made of highly transparent polished, molded plastic, with eight rows of twelve "U" wells and a working capacity of 0.125 ml. Microtiter-calibrated pipette droppers (25 and 50 μl) were used to measure all volumes, and microdiluters with a precisely slotted tip, calibrated to pick up 25 μl were used to carry out serial dilutions on the antigens. The volume content of all the twelve cells is given in Table I.

TABLE I

COMPLEMENT FIXATION INHIBITION ASSAYS USING MICROTITER EQUIPMENT CONTENT OF "U" WELLS

The quantities are expressed as μl.

Reagents	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
									Controls			
									Ag.	*Ab.*	*(Ab+Ag)*	*C'*
Diluent	—	25	25	25	25	25	25	25	25	25	50	100
Antigen	50	←			*dilution*			→	—	25	—	—
Antibody	25	25	25	25	25	25	25	25	25	—	—	—
Complement	50	50	50	50	50	50	50	50	50	50	50	—
Sensitized erythrocytes	25	25	25	25	25	25	25	25	25	25	25	25

Serial dilutions were prepared, a 1 in 2 dilution occurring in each well from 2 to 8. 25 μl of the appropriately diluted antibody were then added to the contents of wells 1–8 and the appropriate controls. 50 μl of complement diluted 1 in 100 were then added to wells 1–11. Each of these plates was sealed and shaken in a constant manner. After this period 25 μl of the appropriately diluted, sensitized red cells were added to each well and the plates were again sealed and shaken as above, and incubated at 37 °C for 60 min. Plates were removed after this period and allowed to sit at 4 °C for 4 h to sediment the cells. The degree of haemolysis was determined visually by observing the amount of sediment in each U well.

In the control experiments using the micro-technique of Wasserman and Levine[20], volumes used in the test tubes were as given in Table II. In this respect, in terms of the dilution of complement and erythrocytes, and the determination of the degree of haemolysis, the procedure was as used by the above workers. Only in the diluting of the antibody and antigen were the procedures changed to fit in with those used in the microtiter experiments.

TABLE II

MICRO-COMPLEMENT FIXATION: CONTENTS (ml) OF THE 12 TUBES REQUIRED

Reagents	*1*	*2*	*3*	*4*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	
								Controls				
Diluent	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.5	0.4	0.4	0.6
Antigen	0.1	0.1	0.1	0.1	0.1	0.1	0.1	—	0.1	—	—	—
Antibody	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1				
Complement (1/250)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	—
Sensitized erythrocytes	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

RESULTS

An homogenate of ox heart, when run on vertical starch gel electrophoresis, was found to contain the multiple esterase forms which were classified by Holmes *et al.*[5], as cholinesterases with relative mobilities (R.M.) 4 and 7, carboxylesterase, R.M. 27 ,32, 36 and 40, arylesterase, R.M. 70 and cholinesterase, R.M. 71. This homogenate was then incubated with the antisera to bovine serum albumin, ox testis acetylesterase, sheep carboxylesterase, chicken carboxylesterase, horse serum cholinesterase and ox arylesterase, respectively; and these combinations were then run on vertical starch gel electrophoresis with the original homogenates. The results of these runs are illustrated in Table III. Antisera bovine serum albumin had no effect on the pattern described above. The antisera to sheep carboxylesterase caused the four bands of carboxylesterases to disappear, and these same four bands were slightly decreased in intensity by the antisera to chicken carboxylesterase. Antisera to horse cholinesterase significantly diminished the intensity of both the slower and faster moving bands of cholinesterase activity. The antisera to ox serum arylesterase caused the fast moving band of arylesterase activity to be completely eliminated.

A similar study carried out on the ox testis homogenate (Table III) showed

similar results for the esterase multiple forms that were present in both bovine tissues. The single slow moving cholinesterase present in this tissue, was again diminished by the anti-cholinesterase sera. A series of more than 10 acetylesterases were present in the testis homogenates, the source of the purified acetylesterase, and the antisera effectively eliminated all of these. Three additional carboxylesterases (classified R.M. 46, 50 and 54 by Holmes and Masters[3]) were absent in the sample incubated with antisera to sheep carboxylesterase, but only slightly diminished in activity in

TABLE III

EFFECT OF ANTISERA ON THE ESTERASE ZYMOGRAMS OF SPECIFIC TISSUE HOMOGENATES

Homogenates were incubated with the indicated specific antisera, and the resultant degree of diminution of activity of individual bands noted. This is indicated as: +++, complete disappearance; ++, moderate decrease; +, slight decrease; −, no effect; B.S.A., bovine serum albumin; O.T.Ac., ox testis acetylesterase; S.L.Ca., sheep liver carboxylesterase; C.L.Ca., chicken liver carboxylesterase; H.S.Ch., horse serum cholinesterase; O.S.Ar., ox serum arylesterase.

Esterases from	*Effect of antisera specific for*					
	B.S.A.	*O.T.Ac.*	*S.L.Ca.*	*C.L.Ca.*	*H.S.Ch.*	*O.S.Ar.*
Ox heart homogenate						
Cholinesterase R.M. 4	−	−	−	−	++	−
Cholinesterase R.M. 7	−	−	−	−	++	−
Carboxylesterase R.M. 27	−	−	+++	+	−	−
Carboxylesterase R.M. 32	−	−	+++	+	−	−
Carboxylesterase R.M. 36	−	−	+++	+	−	−
Carboxylesterase R.M. 40	−	−	+++	+	−	−
Arylesterase R.M. 70	−	−	−	−	−	+++
Cholinesterase R.M. 71	−	−	−	−	++	−
Ox testis homogenate						
Acetylesterase R.M. 5	−	+++	−	−	−	−
Cholinesterase R.M. 7	−	−	−	−	++	−
Cholinesterase R.M. 8	−	+++	−	−	−	−
Cholinesterase R.M. 10	−	+++	−	−	−	−
Cholinesterase R.M. 12	−	+++	−	−	−	−
Cholinesterase R.M. 14	−	+++	−	−	−	−
Carboxylesterase R.M. 46	−	−	+++	+	−	−
Carboxylesterase R.M. 50	−	−	+++	+	−	−
Carboxylesterase R.M. 54	−	−	+++	+	−	−
Arylesterase R.M. 70	−	−	−	−	−	+++
Sheep testis homogenate						
Cholinesterase R.M. 12	−	−	−	−	++	−
Cholinesterase R.M. 18	−	−	−	−	++	−
Carboxylesterase R.M. 21	−	−	−	−	++	−
Acetylesterase R.M. 7	−	+++	−	−	−	−
Acetylesterase R.M. 9	−	+++	−	−	−	−
Acetylesterase R.M. 12	−	+++	−	−	−	−
Acetylesterase R.M. 15	−	+++	−	−	−	−
Acetylesterase R.M. 18	−	+++	−	−	−	−
Arylesterase R.M. 61	−	−	−	−	−	++
Sheep muscle homogenate						
Carboxylesterase R.M. 21	−	−	+++	−	−	−
Carboxylesterase R.M. 39	−	−	+++	−	−	−
Carboxylesterase R.M. 42	−	−	+++	−	−	−
Carboxylesterase R.M. 44	−	−	+++	−	−	−
Carboxylesterase R.M. 46	−	−	+++	−	−	−
Arylesterase R.M. 61	−	−	−	−	−	++

TABLE III *continued*

Esterases from	*Effect of antisera specific for*					
	B.S.A.	*O.T.Ac.*	*S.L.Ca.*	*C.L.Ca.*	*H.S.Ca.*	*O.S.Ar.*
Rat kidney homogenate						
Carboxylesterase R.M. 21	−	−	++	++	−	−
Carboxylesterase R.M. 24.5	−	−	++	++	−	−
Carboxylesterase R.M. 27.5	−	−	++	++	−	−
Carboxylesterase R.M. 31	−	−	++	++	−	−
Acetylesterase R.M. 43	−	+++	−	−	−	−
Acetylesterase R.M. 46	−	+++	−	−	−	−
Acetylesterase R.M. 48	−	+++	−	−	−	−
Pig kidney homogenate						
Cholinesterase R.M. 0	−	−	−	−	++	−
Arylesterase R.M. 22	−	−	−	−	−	++
Acetylesterase R.M. 24	−	++	−	−	−	−
Acetylesterase R.M. 27	−	++	−	−	−	−
Carboxylesterase R.M. 18	−	−	+++	−	−	−
Carboxylesterase R.M. 26	−	−	+++	−	−	−
Carboxylesterase R.M. 28	−	−	+++	−	−	−
Carboxylesterase R.M. 31	−	−	+++	−	−	−
Carboxylesterase R.M. 34	−	−	+++	−	−	−
Arylesterase R.M. 55	−	−	−	−	−	++
Horse kidney homogenate						
Cholinesterase R.M. 4	−	−	−	−	+++	−
Carboxylesterase R.M. 16	−	−	+	+	−	−
Carboxylesterase R.M. 20	−	−	+	+	−	−
Carboxylesterase R.M. 23	−	−	+	+	−	−
Carboxylesterase R.M. 26	−	−	+	+	−	−
Carboxylesterase R.M. 48	−	−	+	+	−	−
Arylesterase R.M. 58	−	−	−	−	−	++
Arylesterase R.M. 60	−	−	−	−	−	++
Guinea pig testis homogenate						
Acetylesterase R.M. 10	−	+++	−	−	−	−
Acetylesterase R.M. 13	−	+++	−	−	−	−
Acetylesterase R.M. 16	−	+++	−	−	−	−
Acetylesterase R.M. 19	−	+++	−	−	−	−
Acetylesterase R.M. 22	−	+++	−	−	−	−
Carboxylesterase R.M. 40	−	−	+++	+	−	−
Carboxylesterase R.M. 44	−	−	++	+	−	−
Arylesterase R.M. 52	−	−	−	−	−	+
Guinea pig kidney homogenate						
Acetylesterase R.M. 10	−	+++	−	−	−	−
Acetylesterase R.M. 13	−	+++	−	−	−	−
Acetylesterase R.M. 16	−	+++	−	−	−	−
Acetylesterase R.M. 19	−	+++	−	−	−	−
Acetylesterase R.M. 22	−	+++	−	−	−	−
Carboxylesterase R.M. 11	−	−	+++	+	−	−
Carboxylesterase R.M. 15	−	−	+++	+	−	−
Carboxylesterase R.M. 20	−	−	+++	+	−	−
Carboxylesterase R.M. 23	−	−	+++	+	−	−
Cholinesterase R.M. 30	−	−	−	−	++	−
Cholinesterase R.M. 33	−	−	−	−	++	−
Carboxylesterase R.M. 40	−	−	+++	+	−	−
Carboxylesterase R.M. 44	−	−	++	+	−	−
Carboxylesterase R.M. 47	−	−	++	+	−	−
Arylesterase R.M. 52	−	−	−	−	−	+
Arylesterase R.M. 63	−	−	−	−	−	+

TABLE II *continued*

Esterases from	*Effect of antisera specific for*					
	B.S.A.	*O.T.Ac.*	*S.L.Ca.*	*C.L.Ca.*	*H.S.Ca.*	*O.S.Ar.*
Rat intestine homogenate						
Cholinesterase R.M. 2	–	–	–	–	++	–
Carboxylesterase R.M. 14.50	–	–	++	+	–	–
Carboxylesterase R.M. 19.0	–	–	++	+	–	–
Carboxylesterase R.M. 37	–	–	+	–	–	–
Carboxylesterase R.M. 39	–	–	+	–	–	–
Carboxylesterase R.M. 42	–	–	+	–	–	–
Cholinesterase R.M. 60	–	–	–	–	++	–
Cholinesterase R.M. 63	–	–	–	–	++	–
Cholinesterase R.M. 67	–	–	–	–	++	–
Cholinesterase R.M. 70	–	–	–	–	++	–
Cholinesterase R.M. 73	–	–	–	–	++	–
Cholinesterase R.M. 83	–	–	–	–	++	–
Cholinesterase R.M. 88	–	–	–	–	++	–
Rat serum						
Cholinesterase R.M. 3	–	–	–	–	++	–
Cholinesterase R.M. 5	–	–	–	–	++	–
Cholinesterase R.M. 8	–	–	–	–	++	–
Carboxylesterase R.M. 48	–	–	++	–	–	–
Carboxylesterase R.M. 52	–	–	++	–	–	–
Carboxylesterase R.M. 65	–	–	++	–	–	–
Arylesterase R.M. 80	–	–	–	–	–	++
Chicken liver homogenate						
Acetylesterase R.M. 17	–	+++	–	–	–	–
Acetylesterase R.M. 21	–	+++	–	–	–	–
Acetylesterase R.M. 25	–	+++	–	–	–	–
Acetylesterase R.M. 28	–	+++	–	–	–	–
Arylesterase R.M. 65	–	–	–	–	–	+
Arylesterase R.M. 69	–	–	–	–	–	+
Carboxylesterase R.M. 53.5	–	–	+	+++	–	–
Carboxylesterase R.M. 57	–	–		+++	–	–
Carboxylesterase R.M. 62	–	–	+	+++	–	–
Carboxylesterase R.M. 66	–	–	+	+++	–	–
Carboxylesterase R.M. 70	–	–	+	+++	–	–
Chicken brain homogenate						
Cholinesterase R.M. 5	–	–	–	+	+++	–
Acetylesterase R.M. 21	–	+++	–	–	+++	–
Acetylesterase R.M. 25	–	+++	–	–	–	–
Acetylesterase R.M. 28	–	+++	–	–	–	–
Arylesterase R.M. 65	–	–	–	–	–	++
Arylesterase R.M. 69	–	–	–	–	–	++
Arylesterase R.M. 73	–	–	–	–	–	++
Arylesterase R.M. 86	–	–	–	–	–	++
Carboxylesterase R.M. 66	–	–	–	+++	–	–
Carboxylesterase R.M. 70	–	–	–	+++	–	–
Chicken spleen homogenate						
Cholinesterase R.M. 5	–	–	–	+	++	–
Arylesterase R.M. 79	–	–	–	–	–	++
Carboxylesterase R.M. 57	–	–	–	+++	–	–
Carboxylesterase R.M. 66	–	–	–	+++	–	–
Carboxylesterase R.M. 66	–	–	–	+++	–	–
Carboxylesterase R.M. 70	–	–	–	+++	–	–

the homogenate sample containing antisera to chicken carboxylesterase. Antisera to bovine serum albumin again had no effect.

Also in Table III, the effect of these antisera on sheep testis and muscle are indicated. These two tissues mutually contained the carboxylesterase classified with R.M. 21 and the arylesterase with R.M. 61. In both tissue homogenates, the presence of anti sheep carboxylesterase sera completely eliminated this carboxylesterase and four others found in the muscle (R.M. 39, 42, 44 and 46). These carboxylesterases were unaffected by any other antisera except that against chicken carboxylesterase, which caused a slight diminution in the intensity with which these bands stained for esterase activity. The arylesterase with R.M. 61 interacted only with the antisera to ox serum arylesterase. In the sheep testis this band was almost completely absent after incubation, while in the muscle it was only about 50% as active as in the original homogenate.

10 esterase multiple forms were distinguishable in the homogenate of pig kidney (Table III) after vertical starch gel electrophoresis, and these corresponded to those found to be cholinesterase R.M. 0, arylesterase R.M. 22, carboxylesterase R.M. 18, 26, 28.5, 31 and 34, acetylesterase R.M. 24 and 27, and arylesterase R.M. 55 on polyacrylamide studies[3]. The cholinesterase was found to be unaffected by all antisera except that against horse serum cholinesterase, which decreased the intensity of staining. Likewise, both arylesterases were effectively diminished only by the antisera specifically prepared against arylesterase. The two acetylesterases were completely absent in the homogenates which had been treated with antisera to ox acetylesterase. All five carboxylesterases were completely eliminated by antisera to sheep carboxylesterase, but appeared to be present with normal activity in homogenates treated with antisera chicken liver carboxylesterase.

Eight of the esterase isoenzymes of horse kidney and the effect of antisera on these are also illustrated in Table III. Cholinesterase (R.M. 4) was completely removed by the corresponding antisera to cholinesterase, but was unaffected by treatment with other sera. The five carboxylesterases (R.M. 16, 20, 23, 26 and 48)[4] were all slightly diminished by both sheep and chicken liver carboxylesterase antisera. Both arylesterases (R.M. 58 and 60) were largely inactivated by treatment with anti arylesterase serum.

Two tissues of the guinea pig, kidney and testis (Table III) were studied in order to cover as many as possible of the isoenzymes classified for the guinea pig. 16 multiple forms were distinguishable in the kidney and 14 in the testis. In the kidney, 6 of the forms were acetylesterases, and 11 of the forms found in the testis were also acetylesterases (R.M. 10–22)[1]. All of these were totally absent when the anti ox acetylesterase sera was added (Fig. 4) but no other sera had any effect. Three distinguishable types of carboxylesterase activity were found in the kidney. One group, containing three bands (R.M. 11, 15 and 20) was totally inhibited by antisera to sheep carboxylesterase, but only slightly diminished by the antisera to chicken carboxylesterase. A single band with relative mobility of 40 was similarly affected. The two bands in the third group (R.M. 44 and 47) were only about 50% diminished by the antisera to sheep liver carboxylesterase and very slightly decreased by anti chicken carboxylesterase sera. The two cholinesterase bands (R.M. 30 and 33) were not completely removed by the addition of anti cholinesterase serum, but were inactivated to some extent by this sera, while none of the other sera had any effect at all. Both arylesterases (R.M. 52

and 63) were also only slightly inhibited by the antiserum against the ox serum arylesterase.

The three tissues studied in the rat were kidney, intestine and serum (Table III). In the kidney only two groups of esterases were found; one containing four carboxylesterases (R.M. 21, 24.5, 27.5 and 31)[2] and the other containing three acetylesterases (R.M. 43, 46 and 48). All four carboxylesterases were diminished to approximately one half of their original intensity by the antisera to carboxylesterases of both sheep and chicken liver, and the acetylesterase was totally eliminated by the anti acetylesterase serum. The rat intestine homogenate was differentiated into 13 esterase multiple forms, 8 of which were cholinesterase, one very slow moving (R.M. 2) and the other 7 faster moving species (R.M. 60, 63, 67, 70, 73, 73 and 88). All of these were effectively inhibited by only one antiserum: that prepared against horse serum cholinesterase. The other five isoenzymes consisted of two groups of carboxylesterase. The slower group containing two forms with relative mobilities 14 and 19, were almost completely eliminated by anti sheep carboxylesterase serum, but only very slightly decreased by incubation with anti chicken carboxylesterase serum. The other group of three carboxylesterases (R.M. 37, 40 and 42) were only slightly affected by the antiserum to sheep carboxylesterase and were not inhibited by the serum to chicken carboxylesterase. In the rat serum, eight esterase multiple forms were studied. Three of these were cholinesterases (R.M. 3, 5 and 8) and were completely inhibited by the antiserum to the horse cholinesterase. Three carboxylesterases were present (R.M. 48, 52 and 56) and although all were unaffected by the antiserum to chicken esterase, the antiserum against sheep esterase diminished the intensity of specific staining in the regions where the carboxylesterases ran in the control. An arylesterase (R.M. 80) was also present, and as with all the other arylesterases was only affected by the antiserum to the ox arylesterase, which appeared to be much more effective in inhibiting this serum arylesterase than any other tissue arylesterase studied.

The liver, brain and spleen of the chicken were also studied (Table III). The six arylesterases of the liver and the three acetylesterases of the brain (R.M. 17 to 38)[3] were all effectively inhibited by the antiserum against the ox acetylesterase. In the brain, three arylesterases were found (R.M. 65, 69 and 73); the two bands with R.M 65 and 69 were also present in the liver, while another form with R.M. 77 was found in the spleen. All of these were unaffected by any antisera except that to ox arylesterase which reduced, but did not eliminate their activity. In the brain and spleen, a cholinesterase with relative mobility of five was totally eliminated by the antiserum to the cholinesterase of horse serum. The antiserum to chicken liver carboxylesterase also had a definite inhibitory effect on the cholinesterase of both these tissues causing a reduction to approximately 2/3 of the original intensity. In the liver the five remaining isoenzymes were carboxylesterases (R.M. 53.5, 57, 62, 66 and 70) and although totally eliminated by anti chicken carboxylesterase serum, these bands were only weakly inhibited by anti sheep carboxylesterase serum. In the brain with three carboxylesterases, and the spleen with four, similar inhibition by antiserum was found.

Complement fixation

Using the system described for the Microtiter apparatus, complement fixation inhibition assays were carried out using various dilutions of pure bovine serum albumin, ox testis acetylesterase, ox serum arylesterase, horse serum cholinesterase, sheep

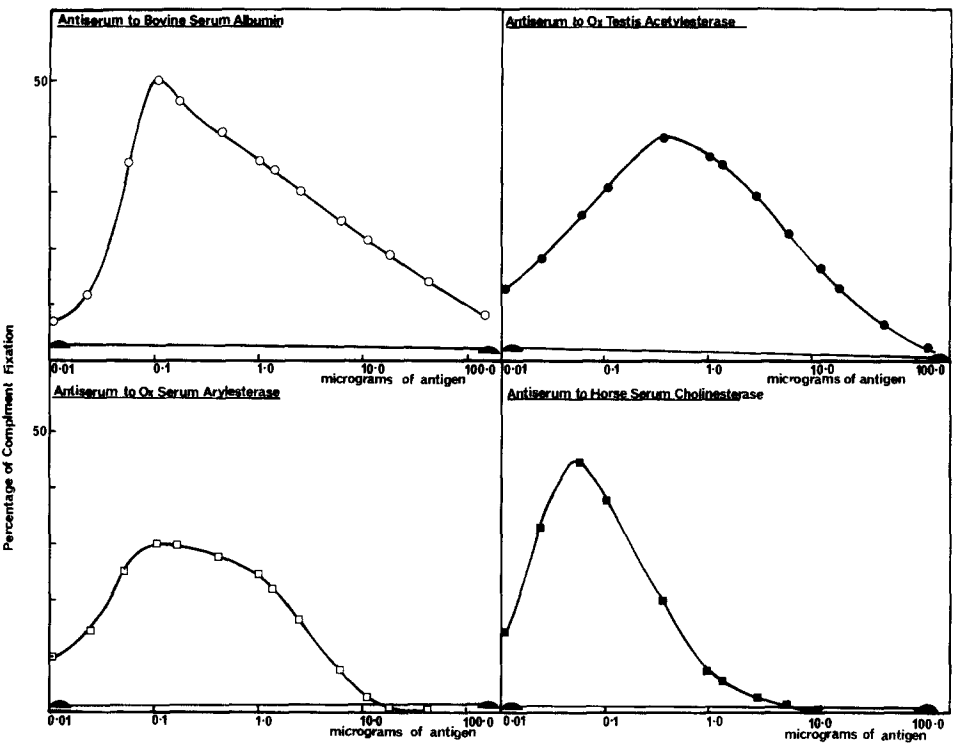

Fig. 1. (a) Complement fixation of anti bovine serum albumin with bovine serum albumin (○). (b) Complement fixation of anti ox testis acetylesterase with ox testis acetylesterase (●). (c) Complement fixation of anti ox serum arylesterase with ox serum arylesterase (□). (d) Complement fixation of anti sheep liver carboxylesterase with sheep liver carboxylesterase (■).

liver carboxylesterase and chicken liver carboxylesterase. Likewise, the antisera to the acetylesterase, the arylesterase, the cholinesterase and the sheep and the chicken carboxylesterases were all tested against the same group of antigens as the serum albumin antiserum. An initial dilution of the anti bovine serum albumin antiserum of 1 in 10 000 was found to give approximately 50% complement fixation as a peak against the albumin (Fig. 1a). This dilution of antisera was maintained in all cases where any fixation occurred at this dilution of antisera (such as in the case of the assays of anti bovine serum albumin sera against ox acetylesterase, ox arylesterase, horse cholinesterase, sheep carboxylesterase and chicken carboxylesterase), the antiserum was re-assayed at a dilution of 1 in 2000; and in the above cases, there was again no complement fixation. In Fig. 1b, the assay of anti acetylesterase serum against acetylesterase is illustrated with a peak of complement fixation of 40%, using 0.5 μg of acetylesterase. Again no complement fixation could be obtained using this antisera diluted to 1 in 2000 against the other antigenic determinants. Similar results were obtained using anti arylesterase sera against its own antigenic determinant, but with a 30% peak of complement fixation. Acetylesterase and arylesterase both peaked within a 10-fold increment of each other; arylesterase at 0.1 μg (Figs 1c and 1d). In the bovine serum albumin assay a much lower titer of 0.01

micrograms gave peak complement fixation. The antisera to sheep liver carboxylesterase peaked to 45% with 0.7 μg of the pure antigen. No complement fixation was observed when the antigens used in the assays were anything other than the sheep liver carboxylesterase.

Between the antisera to the chicken liver carboxylesterase and the horse serum cholinesterase, some degree of cross reaction with the alternate antigen was found (Fig. 2). When assayed with chicken carboxylesterase, the antisera to this enzyme gave a sharp peak of activity of 50% complement fixation using 1.0 μg of esterase. When horse serum cholinesterase was substituted as the antigen, about 8% complement fixation occurred with the antisera still at a dilution of 1 in 10 000, and using a similar amount of antigen. No interaction between this antisera to the chicken carboxylesterase and any of the other four antigens was found (Fig. 2a and 2b). Similar results in reverse (Figs 2c and 2d) were found when anti horse serum cholinesterase was assayed against horse cholinesterase and chicken liver carboxylesterase, and inhibition of haemolysis assayed. With its natural antigen, this sera gave approximately 40% inhibition of haemolysis with 0.25 μg of antigen. At a similar concentration of antigen, only 10% complement fixation was obtained for the antigen chicken carboxylesterase.

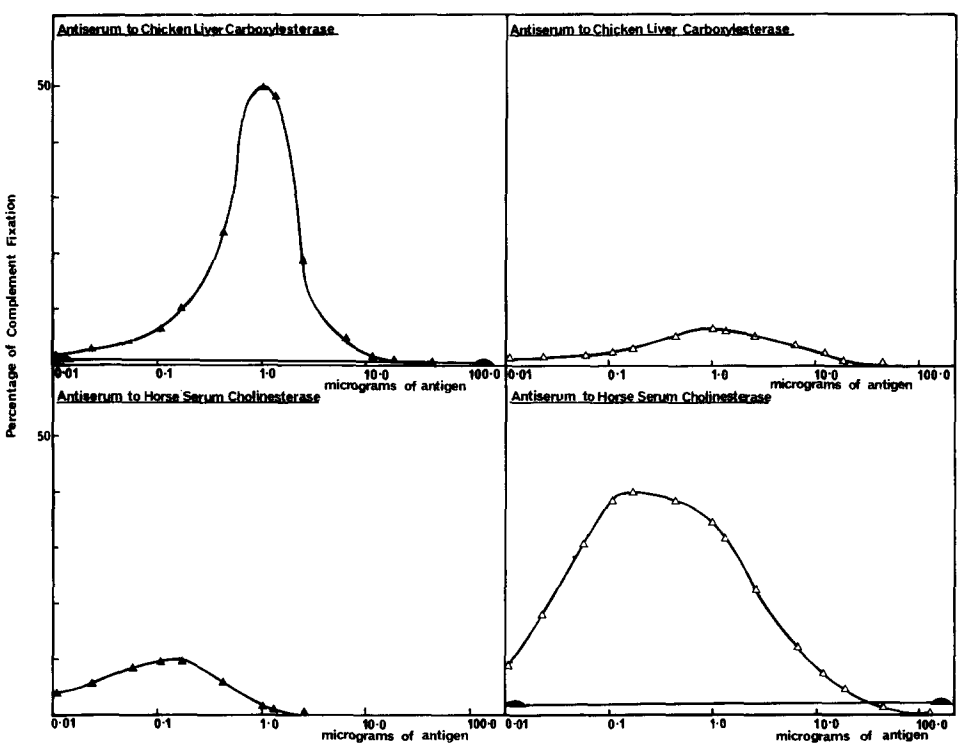

Fig. 2. (a) Complement fixation of anti chicken liver carboxylesterase with chicken liver carboxylesterase (▲). (b) Complement fixation of anti chicken liver carboxylesterase with horse serum cholinesterase (△). (c) Complement fixation of anti horse serum cholinesterase with chicken liver carboxylesterase (▲). (d) Complement fixation of anti horse serum cholinesterase with horse serum cholinesterase (△).

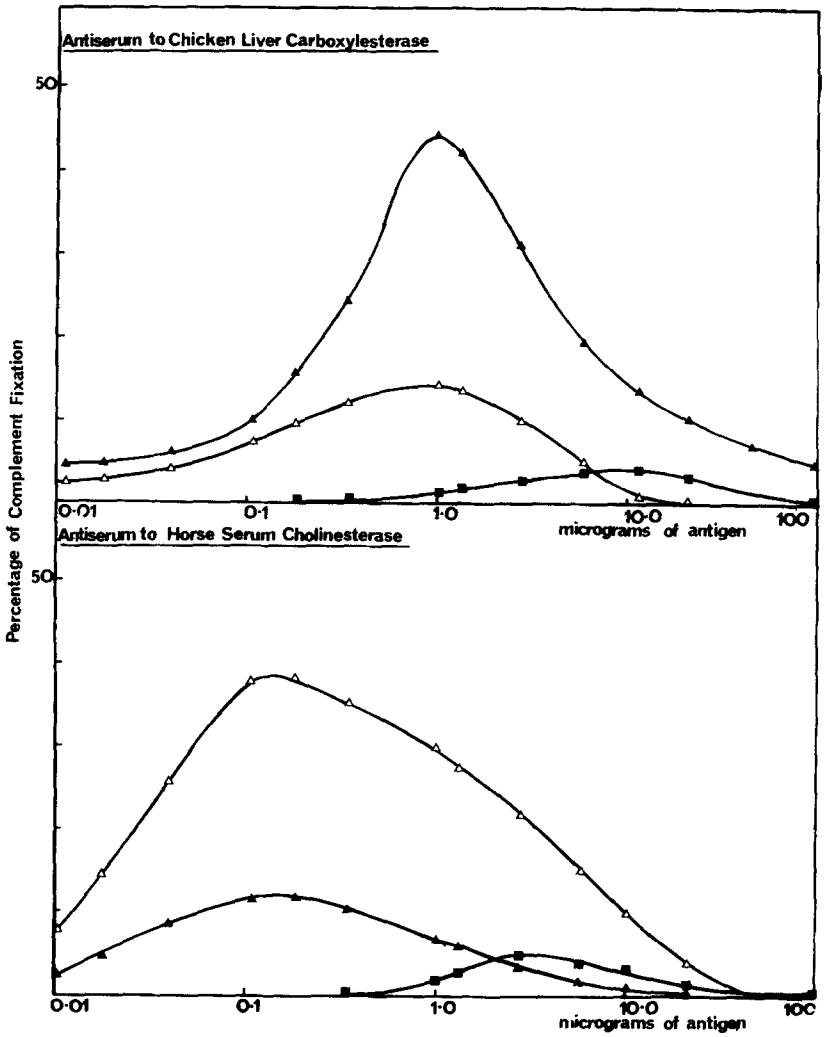

Fig. 3. (a) Complement fixation of anti chicken liver carboxylesterase with: ▲, chicken liver carboxylesterase; △, horse serum cholinesterase; ■, sheep liver carboxylesterase. (b) Complement fixation of anti horse serum cholinesterase with: △, horse serum cholinesterase; ▲, chicken liver carboxylesterase; ■, sheep liver carboxylesterase.

Then in order to provide further confirmation of the principal features of these results, the quantitative micro-complement system of Wasserman and Levine[20] was applied and the data in Fig. 3 resulted. At a dilution of 1 in 10 000 the antiserum to chicken carboxylesterase gave 44% complement fixation at 1.0 μg of chicken liver carboxylesterase, and with horse serum cholinesterase the same quantity of protein gave 14% complement fixation. Using sheep carboxylesterase as an antigen, some fixation occurred with 10.0 μg. This was less than 5%, but was distinctive, and clearly more than that found with the other three antigens which gave no inhibition to the haemolysis. The antiserum to horse serum cholinesterase at a dilution of 1 in

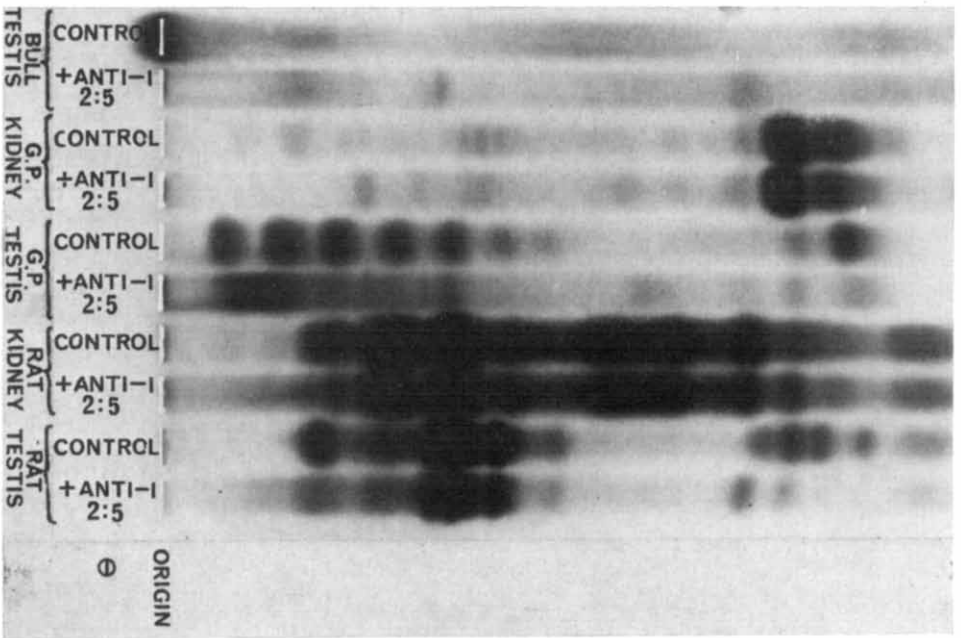

Fig. 4. Starch gel zymograms of esterase activity in mammalian tissues, and the comparative effect of preincubation with antisera against the acetylesterase from bull testis (anti-1).

10 000, gave 39% complement fixation with 0.25 μg of horse serum cholinesterase and 12% fixation with the same amount of chicken carboxylesterase. Sheep carboxylesterase was the only other antigen of those tested to give a fixation peak. This peak of about 5% occurred with 4 μg of sheep carboxylesterase.

DISCUSSION

As a first comment on this immunological approach to the problems of esterase inter-relationships, the proven value of these parameters in comparative studies of protein structure may be noted[30–32]. It is to be expected, of course, that immunological cross-reactivity would be especially informative as an indication of similarity between proteins at the level of the respective surface determinant groups.

With the acetylesterases, for example, the antiserum to the ox testis enzyme was extremely efficient in removing all the acetylesterase forms from the homogenates with which it was incubated. Since the purified acetylesterase represents a single species from a tissue containing upward of 13 multiple forms of this type of activity, the present evidence may be viewed as consistent with other indications that the multiplicity of the ox testis acetylesterase is due to epigenetic modifications of a single protein species (N. E. Haites, unpublished results). Furthermore, it would appear that the main form of the enzyme of ox testis is not only immunologically very similar to all the other acetylesterase heteromorphs of that species, but also of a similar nature to the acetylesterases in the other species studied. It is also significant

that the acetylesterases are immunologically quite distinct from the other types of soluble esterolytic activity (*e.g.* carboxylesterases, cholinesterases and arylesterases), which are present in mammalian tissues.

In the case of the antisera to sheep liver carboxylesterase and chicken liver carboxylesterase, these proved capable of interacting with all the bands of carboxylesterase activity in the species from which the pure antigen was obtained. Thus, although sheep testis and muscle contained two groups of esterases which are differentiatable on the basis of their physical properties[3], all of these forms are sufficiently similar to be distinguishable by this immunological technique. Similarly, all of the carboxylesterases found in the ox heart and testis and in the pig kidney, and two of the three groups of carboxylesterases found in the guinea pig kidney and testis, were also indicated as immunologically closely related. A third group of guinea pig carboxylesterases, those two bands classified with relative mobilities 44 and 47, however, were affected by the antiserum to a markedly lesser degree than the other two groups of this species. Since these particular carboxylesterases are similar in all the other properties that have been reported previously[1], it seems likely that the immunologically distinct pair are not grossly dissimilar in protein structure to the other carboxyesterases of the guinea pig, nor to those of the sheep, ox and pig. The carboxylesterases of the rat tissues, and to a lesser extent, those of the horse kidney are also indicated as related. This variation in the degree of interaction of the antisera with the carboxylesterases of different animals is consistent with the presence of multiple loci in some mammalian species, and the divergent evolution of these forms of esterase activity (refs 1–7 and 22; K. Scott, M. T. C. Runnegar and B. Zerner, personal communication; P. Inkerman and B. Zerner, personal communication).

In the case of the cholinesterases, the ability of an antiserum produced against a horse serum enzyme to interact with the tissue esterases of the horse kidney, chicken brain and spleen would seem to indicate a close structural relationship between the tissue and serum cholinesterases, despite the many reports of considerable molecular weight differences between these sources[13]. Some slight reduction in activity occurred with the tissue cholinesterases of all other species, as well, pointing to a general chemical relatedness of these forms of esterase activity.

Of the vertebrate tissue arylesterases whose molecular weights have been reported previously, all have had molecular weights between 50 000 and 70 000 (ref. 6). The arylesterase purified from ox serum by Kitchen[8], however, has been shown to be a dimeric lipoprotein with a mol. wt greater than 140 000, and a marked change in conformation attends delipidation of this enzyme. It is not surprising, therefore, that the antisera to this ox serum arylesterase, although completely eliminating the arylesterases of ox heart and testis, only partially diminished the arylesterase activity of the tissues of all other species studied. A strong structural similarity to the ox tissue arylesterases with appreciable structural differences between species is indicated by the immunoelectrophoretic evidence.

Of all the mammalian esterases, the most intensively studied types of activity to date have been the liver carboxylesterases and the serum cholinesterases. In the horse, pig and sheep, the amino acids surrounding the active site of the carboxylesterases have been shown to be identical for at least eight amino acids (Gly–Glu–Ser–Ala–Gly–Gly–Glu–Ser–)[23,26], while the ox, and chicken carboxylesterases differ by only a single amino acid substitution. This data may be taken as indicating that

the carboxylesterases of the horse, pig and sheep are more closely related to each other than to those of ox and chicken. In regard to this relationship, it may be noted that the antiserum to chicken carboxylesterase has only a slight inhibitory effect on any carboxylesterase of another species, and caused no diminution in the bands of the pig kidney, rat serum or sheep tissue carboxylesterases. Although showing little immunological relationship with the carboxylesterases of other species, this antiserum was unique among those studied, in that it inhibited the staining of an esterase form other than the natural antigen type, reducing the activity of the chicken cholinesterase band appreciably. In this connexion it may be remarked that studies on the active site of the horse cholinesterase have shown a tripeptide (-Glu–Ser–Ala-)[13,23], similar to that found in the carboxylesterases at the active serine. Hence, even though carboxylesterases and cholinesterases have different substrate and inhibitor characteristics, and exhibit different size properties in vertebrate tissues[25,28], the basic sequences at the active site may be taken as indicating some similarities in structure; and this conclusion is supported at the level of surface determinants by the immunological interaction between the chicken carboxylesterase and the chicken cholinesterase.

As a confirmatory approach to these immunoelectrophoretic findings, the immunological relationships of the esterases were investigated by micro-complement fixation; a technique which is established as extremely sensitive to differences in protein structure[29,30]. Both in the microtiter system and in the quantitative micro-complement fixation results, the antisera to chicken liver carboxylesterase and horse serum cholinesterase showed interaction with each of these antigenic determinants. Again, both of these sera reacted with sheep carboxylesterase in these systems, but much higher amounts of this antigen were required to form the antigen–antibody complex, and the maximum fixation was low. Taken together, then, these findings appear to possess considerable significance in relation to the phylogenetic interrelationships of the esterases, in that they confirm that there are similarities between the carboxylesterases and the cholinesterases. These similarities are strong in the chicken, but less appreciable between the sheep liver carboxylesterase, the chicken liver carboxylesterase and the horse serum cholinesterase.

The present data, overall, also allow comment in a more general context: that is in regard to the extent of inter-relationships within the esterase heteromorph systems as compared to those examples of enzyme multiplicity where structural details have been intensively studied. The A and B subunits of lactate dehydrogenase, for example, give no cross complement fixation, and likewise, there is little or no cross reaction between the lactate dehydrogenases of different species[31]. In contrast, not only did the multiple forms of carboxylesterases, the acetylesterases, the cholinesterases and the arylesterase behave similarly within each enzyme system of a single species, but some cross reaction was also noticeable within the same esterase type between species. Consequently, it would appear that the relationships between the individual esterase types in different species is closer than that between the parental forms of lactate dehydrogenase in a single species. Again, it is significant that the classification of the vertebrate esterases, based on relative mobilities, and substrate and inhibitor specificities, is consistent with and has been substantiated by these immunological data.

In summary, then, this study has shed further light on relationships between

the different esterase multiple forms, both within individual species and between species. In all of the species studied, the acetylesterases were indicated by the immunological criteria as having closely related structural characteristics. Similarly, within single species, all the multiple forms of this type of activity behaved in a similar manner, providing support for the interpretation that epigenetic influences may be the cause of their multiple presence. The carboxylesterases within single species were again indicated as structurally similar to one another, but with these enzymes, much greater differences were observable between species. While the horse and the chicken carboxylesterases appeared to be immunologically distinct, the relationship between the carboxylesterases and the cholinesterases in the chicken indicated that these enzymes may have formed into separate entities from one genetic precursor, quite recently in evolutionary terms. Although both the cholinesterases and the arylesterases, separately, showed close relationships between their multiple forms within a species, each species examined retained a distinct immunological identity with respect to both of these types of esterase activity.

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