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Genetic control of the hydrolysis of aromatic esters by sheep plasma A-esterase

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1. INTRODUCTION

Lee (1964) described two types of sheep, one of which possessed a high level of esterase activity against di-(2-chloroethyl) aryl phosphates, the other a low level.

The low level sheep were shown to breed true, and the suggestion was that these were one homozygous class of the three classes which would be expected from a two-allele system segregating in simple Mendelian fashion. However, no distinction into two groups was possible for those sheep which hydrolysed haloxon rapidly, and it was proposed that either the homozygous group, with extremely rapid haloxon hydrolysis, had not been encountered, or that complete dominance existed.

In order to resolve this alternative, further work has been carried out to determine whether other substrates would reveal different levels of esterase activity in sheep plasma and, if so, whether these differences would support the proposed genetic mechanism for the inheritance of ability to hydrolyse haloxon. In addition, these biochemical tests have been performed on plasma from sheep of known hereditary relationship to ascertain whether the phenotypes of the offspring were in accordance with the genetic possibilities of the proposed genotypes of the parents. These genotypes were derived from their pattern of plasma esterase activity.

2. EXPERIMENTAL

(i) *Materials*

Two flocks of sheep were used in these experiments. The first was a flock of 105 Welsh Mountain ewes picked at random from stock. The second was a selected flock of 20 Clun Forest and Welsh Mountain ewes, which had previously been shown to be active in the hydrolysis of di-(2-chloroethyl) aryl phosphates (Lee, 1964), together with their offspring. The sire of the breeding flock was a Clun Forest ram, also with high esterase activity, purchased from the flock maintained by the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge.

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Ewes and lambs were run together on pasture throughout the course of the experiment, the lambs being marked at birth with paint for ease of identification until they were ear-tagged at approximately one month old.

Blood samples were taken by needle puncture of the jugular vein, clotting being prevented by heparin. Plasma was obtained by centrifuging the blood samples at approximately 4000 *g* for 20 min.

The esterase activity of the plasma samples was tested using the following esters as substrates:

Indophenyl acetate (IA). Cooper Technical Bureau, prepared according to the method of Archer & Zweig (1959).

1-Naphthyl acetate (NA). British Drug Houses, Ltd.

1-Naphthyl butyrate (NB). Cooper Technical Bureau.

4-Nitrophenyl butyrate (NPB). Cooper Technical Bureau.

4-Ethoxycarbonylcoumarin-7-yl acetate. Cooper Technical Bureau.

Diethyl 3-chloro-4-methylcoumarin-7-yl phosphate (Coroxon). Cooper Technical Bureau.

Di-(2-chloroethyl)-3-chloro-4-methylcoumarin-7-yl phosphate (haloxon). Cooper Technical Bureau.

(ii) *Methods*

All hydrolyses were carried out in 0·04 M phosphate buffer maintained at 37°C. by a water bath. Table 1 gives the relevant details of conditions of hydrolysis for each substrate. In each case the action of the enzyme produces an acid and a phenol, the quantitative production of the latter being used to determine the rate of hydrolysis.

The indophenol produced under the conditions in Table 1 was estimated by measuring the absorption of the solution at 625 mμ (Kramer & Gamson, 1958) and subtracting the absorption due to non-enzymic hydrolysis. The μmoles of indophenol were read off from a standard curve prepared by plotting the absorption at 625 mμ of known quantities of indophenol.

Similarly, 4-nitrophenyl butyrate and 4-ethoxycarbonylcoumarin-7-yl acetate are also chromogenic substrates, and the 4-nitrophenol and 4-ethoxycarbonyl-7-

Table 1. *Conditions employed for the hydrolysis of various esters by sheep plasma*

Substrate	pH	Minutes of incubation	Plasma (ml.)
Indophenyl acetate ($2{\cdot}5 \times 10^{-4}$ M)	8·0	15	0·05
1-Naphthyl acetate (6×10^{-4} M)	7·4	10	0·00125
1-Naphthyl butyrate (6×10^{-4} M)	7·4	15	0·00125
4-Nitrophenyl butyrate (10^{-3} M)	7·6	10	0·01
4-Ethoxycarbonylcoumarin-7-yl acetate (10^{-4} M)	7·4	20	0·01
Haloxon ($2{\cdot}5 \times 10^{-4}$ M)	7·4	30	0·1
Coroxon ($2{\cdot}5 \times 10^{-4}$ M)	7·4	30	0·1

hydroxycoumarin produced were determined by reading off the absorption of the solutions at 410 and 390 mμ respectively, and calculating μmoles from a standard curve.

The 1-naphthol, produced by the hydrolysis of the 1-naphthyl acetate and butyrate, was reacted with diazo-blue B dye in the presence of sodium lauryl sulphate (Van Asperen, 1962). It was found that the blue colour which developed could be stabilized by the addition of two drops of 4M HCl. The μmoles of 1-naphthol produced were determined by comparing the absorption at 600 mμ of the test solutions with that of standard amounts of 1-naphthol which had been similarly treated.

The determination of hydrolysis rates of coroxon and haloxon has been previously described (Lee, 1964).

Electrophoresis

Agar gel electrophoresis was carried out according to the method of Wieme (1959) with the following modifications:

(1) The apparatus was a Universal Mark II (Shandon Scientific Co.) as described by Kohn (1960).
(2) The glass slides, with their covering of agar gel (2 mm.) were placed in shallow circular perspex dishes (diam. 9 cm.), which were in turn positioned on the supports of the apparatus.
(3) The bridges leading from the buffer to the gel were of the same agar gel (Oxoid No. 2), with an underlying layer of filter paper to prevent slipping.
(4) Cooling was achieved by filling the perspex dishes with 40–60°C. petroleum spirit, which was continually replenished.

The electrophoretic run took place under constant amperage (usually 30 mA.) which, with the cooling system employed, produced a voltage drop of about 160 V. falling to 150 V. during the 60 min. run.

The plasma (0·02 ml.) was introduced into a slit at the electronegative end of the agar gel slide, and all measurements of mobility were made relative to this slit. The direction of current was reversed after each run to reduce to a minimum any changes which occurred in the buffer in the compartments.

Identification of the bands of esterase activity was achieved by incubating the gel slides in appropriate solutions of substrates and reagents:

(*a*) 1-Naphthyl acetate (10^{-3}M), plus Fast Blue RR salt (Sigma Chemical Co.); black bands appear at sites of esterases which hydrolyse this substrate.
(*b*) 1-Naphthyl butyrate; as for 1-naphthyl acetate.
(*c*) Indophenyl acetate (10^{-3}M); hydrolysis of this substrate produces blue bands of indophenol at the sites of activity.
(*d*) 4-Ethoxycarbonylcoumarin-7-yl acetate (10^{-4}M); hydrolysis of this substrate produces yellow bands of 4-ethoxycarbonyl-7-hydroxycoumarin at sites of esterase activity.

(*e*) Di-(2-chloroethyl) 4-ethoxycarbonylcoumarin-7-yl phosphate; reaction as for the acetate.

(*f*) Acetyl thiocholine iodide (10^{-3}M), plus 5,5′dithiobis-(2-nitro) benzoic acid; yellow bands appear at the sites of esterases which hydrolyse this substrate.

All substrates and reagents were made up in the same Veronal buffer (pH, 8·6; 0·1 μ) which was used for the manufacture of the agar gel slides.

Location of bands was also done by slicing the gel into strips with a cutter made of twenty safety-razor blades (Ever-ready) stuck together. Each strip was approximately 2 mm. wide, and these strips were then incubated in tubes with indophenyl acetate or acetyl thiocholine at 37°C. The optical density at 625 and 410 mμ respectively was read off at 5 and 65 min. after the addition of substrate. A plot of the changes in optical density for each strip gave a permanent record of the electrophoretic run in terms of esterase activity (see Fig. 1).

3. RESULTS AND DISCUSSION

When the breeding flock of sheep was bled, and the plasma tested for esterase activity against haloxon, it was found that the lambs could be divided into three types, one of which had a low rate of haloxon hydrolysis, the second a moderately high rate of hydrolysis, and the third had a very high rate of haloxon hydrolysis which was approximately twice that of the moderately high group (Table 2).

Table 2. *The hydrolysis of esters by six sheep genotypes from two flocks*

Proposed *Es* genotype	No.	IA Mean	IA ± S.E.	CA Mean	CA ± S.E.	NA Mean	NA ± S.E.	Haloxon Mean	Haloxon ± S.E.
a/*a* (B)	9	22	$0·6^{x}$	42	$3·7^{x}$	853	$8·6^{z}$	5·2	$0·3^{z}$
a/*a* (R)	6	19	$0·4^{x}$	39	$7·3^{x}$	847	$28·5^{z}$	5·6	$0·3^{z}$
a/*b* (B)	25	34	$0·5^{y}$	210	$14·5^{y}$	350	$15·0^{y}$	2·7	$0·1^{y}$
a/*b* (R)	28	33	$0·6^{y}$	202	$4·3^{y}$	400	$17·0^{y}$	2·9	$0·1^{y}$
a/*c* (B)	5	16	$1·2^{x}$	39	$2·3^{x}$	358	$24·0^{y}$	3·2	$0·2^{y}$
a/*c* (R)	6	15	$1·7^{x}$	33	$6·0^{x}$	405	$28·0^{y}$	3·2	$0·2^{y}$
b/*b* (B)	2	48	$1·0^{z}$	354	$12·0^{z}$	15	$—^{x}$	0·4	$—^{x}$
b/*b* (R)	44	44	$0·5^{z}$	343	$5·0^{z}$	23	$1·4^{x}$	0·4	$0·02^{x}$
b/*c* (B)	2	36	$1·0^{y}$	207	$3·0^{y}$	12	$2·0^{x}$	0·3	$0·1^{x}$
b/*c* (R)	20	33	$0·8^{y}$	204	$5·0^{y}$	19	$2·0^{x}$	0·2	$0·02^{x}$
c/*c* (R)	1	14	$—^{x}$	26	$—^{x}$	15	$—^{x}$	0·1	$—^{x}$

IA = Indophenyl acetate. CA = 4-ethoxycarbonylcoumarin-7-yl acetate. NA = 1-naphthyl acetate. All activities are given as μmoles/ml./hour. For any one substrate, groups bearing a different superscript letter are significantly different ($P < 0·01$) from each other. The symbols (B) and (R) indicate that the animals came from the Breeding or Random flock, respectively.

However, further work on sheep plasma esterase, using carboxylic esters as substrates, demonstrated that five types could be distinguished according to the pattern of activity against the various substrates used.

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These five types are as follows:

Group 1 hydrolysed 1-naphthyl acetate (NA) very rapidly but 4-ethoxycarbonylcoumarin-7-yl acetate (CA) very slowly.

Group 2 hydrolysed NA moderately well, but CA very poorly.

Group 3 hydrolysed both NA and CA moderately well.

Group 4 hydrolysed NA very poorly, but CA moderately well.

Group 5 hydrolysed NA very poorly, but CA very rapidly.

In all groups, indophenyl acetate (IA) gave the same pattern as CA, and haloxon (HAL), 4-nitrophenyl butyrate (NPB) and 1-naphthyl butyrate (NB) followed the same variations as NA (Tables 2 and 3). By contrast, Coroxon (COR) was hydrolysed at one of two rates (Table 3), the lower rate corresponding to Groups 2 and 4 above.

Table 3. *The hydrolysis rates for naphthyl butyrate, 4-nitrophenyl butyrate and Coroxon by plasma from the six genotypes of sheep shown in Table 2*

Proposed *Es* genotype	No.	NB Mean	NB ± S.E.	NPB Mean	NPB ± S.E.	Coroxon Mean	Coroxon ± S.E.
a/a	15	466	$15{\cdot}0^{x}$	59	$1{\cdot}9^{x}$	1·8	$0{\cdot}1^{x}$
a/b	53	186	$8{\cdot}0^{y}$	35	$3{\cdot}1^{y}$	1·5	$0{\cdot}2^{x}$
a/c	11	166	$4{\cdot}9^{y}$	30	$0{\cdot}7^{y}$	0·9	$0{\cdot}03^{y}$
b/b	46	7	$0{\cdot}3^{z}$	22	$0{\cdot}5^{z}$	1·4	$0{\cdot}2^{x}$
b/c	22	9	$0{\cdot}3^{z}$	20	$0{\cdot}9^{z}$	0·8	$0{\cdot}01^{y}$
c/c	1	4	—^{z}	19	—^{z}	0·1	—^{z}

NB = 1-naphthyl butyrate. NPB = 4-nitrophenyl butyrate. For any one substrate, groups bearing a different superscript letter are significantly different ($P < 0{\cdot}01$) from each other. All activities are given in μmoles/ml./hour.

The two allele hypothesis, put forward by Lee (1964) to account for the variation in haloxon hydrolysis by sheep, does not allow for five phenotypes; however, the existence of a third allele, which produces little or no esterase activity, would provide a solution to this problem, but necessitates the existence of a sixth phenotype, which corresponds to a sheep with low plasma hydrolysis rates for all the substrates used in this study.

In an attempt to find this sixth phenotype, a separate flock of Welsh Mountain ewes was bled and tested for plasma esterase activity against IA, CA, NA and haloxon. The results are summarized in Table 2, and confirm the five types already found in the breeding flock, together with one sheep which hydrolysed none of the substrates, and was, therefore, the sole representative of the sixth group whose existence was postulated from the results obtained with the breeding flock. This particular sheep was re-bled, and tested a second time to ensure that no unusual inactivation had occurred during the first test.

Good agreement for hydrolysis rates was obtained between the two flocks (Table 2), significant differences ($P < 0{\cdot}01$, 't' test) only occurring between phenotypes and not between flocks.

On the basis of these six phenotypes, a three-allele mechanism of heredity is proposed for sheep plasma esterase. The three alleles have been termed Es^a, which governs the production of esterase capable of hydrolysing NA, NB, NPB and haloxon; Es^b, which governs the production of esterase capable of hydrolysing CA and IA; and Es^c, which produces no esterase activity against these substrates. Since only two alleles are present at the locus, six genotypes are possible, each distinguishable by the pattern of its esterase activity. Thus, Es^a/Es^a would have a very high activity against the naphthyl acetate group of substrates, but a very low activity for CA and IA; Es^a/Es^b would have a moderate ability to hydrolyse both groups of substrates. Similarly, Es^a/Es^c, Es^b/Es^b, Es^b/Es^c and Es^c/Es^c would be the genotypes for Groups 2, 5, 4 and 6 respectively in the phenotypic classification.

Each of the groups in Tables 2 and 3 has been assigned a genotype that describes its ability to hydrolyse esters in quantitative terms. In the case of Coroxon, Es^a and Es^b are assumed to confer equal hydrolysing ability on the plasma, since Es^a/Es^a, Es^a/Es^b and Es^b/Es^b genotypes were indistinguishable with this substrate, and these three types had about twice the activity of the Es^a/Es^c and Es^b/Es^c genotypes (Table 3).

Table 4. *Distribution of genotypes in a flock of Welsh Mountain sheep, compared to that expected from a flock with random distribution of genetic material*

	Proposed *Es* genotypes					
	a/a	*a/b*	*a/c*	*b/b*	*b/c*	*c/c*
Nos. of sheep observed*	44	6	1	28	20	6
expected*	44	5	2	30	18	6

* A χ^2 test showed that there is a greater than 95% probability that these observed and expected results are not significantly different.

The Welsh Mountain flock has been termed 'random' in Table 2; this name arose initially from the way in which the animals were selected from a larger flock prior to bleeding. However, when the biochemically assigned allelic distribution was examined for goodness of fit to the Hardy–Weinberg equation for genetic equilibrium (Table 4), it was found that this flock was also random with respect to distribution of genetic material, which is, therefore, in equilibrium at this locus, as previously suggested by Lee (1964).

This lack of selection pressure for a particular esterase pattern makes speculation as to the natural function of these esterases a worthless pursuit on the present evidence.

The results of the mating experiment are given in Table 5; the 22 ewes were divided into four groups by the biochemical tests, including one Es^b/Es^b which was

included by error. The lambs were also classified according to the biochemical evidence, and the resulting distribution of the genotypes was in accord with the proposed genetic hypothesis, although the results were too few to constitute a confirmation of biochemical results.

It is of interest to note that the one set of twins, which resulted from an $Es^a/Es^b \times Es^a/Es^b$ mating, segregated cleanly into one Es^a/Es^a and one Es^b/Es^b. This suggests that these twins were dizygotic, and that sheep esterase activity is not modified by any twinning effects. Unfortunately, both of the twins were female, so that their dizygoticity was not confirmed.

Further evidence that the esterase activity of sheep plasma is governed by alleles at the same gene locus was obtained electrophoretically. Under varying conditions of current and time of electrophoretic run, no difference in mobility was observed for the bands of esterase activity derived from different sheep genotypes and phenotypes. This indicates that the two types of esterase are identical or very similar in

Table 5. *The results of a mating experiment involving sheep of different patterns of plasma esterase activity*

Mating			Offspring *Es* genotypes					
♀ *Es* genotype		♂ *Es* genotype	*a/a*	*a/b*	*a/c*	*b/b*	*b/c*	*c/c*
a/b	×	*a/b*	4	9	0	2	0	0
a/c	×	*a/b*	3	1	0	0	1	0
a/a	×	*a/b*	1	1	0	0	0	0
b/c	×	*a/b*	0	1	0	0	0	0

One $a/b \times a/b$ mating produced twins, all other mating gave rise to single lambs.

electrical properties, which would be expected for proteins governed by the same gene locus.

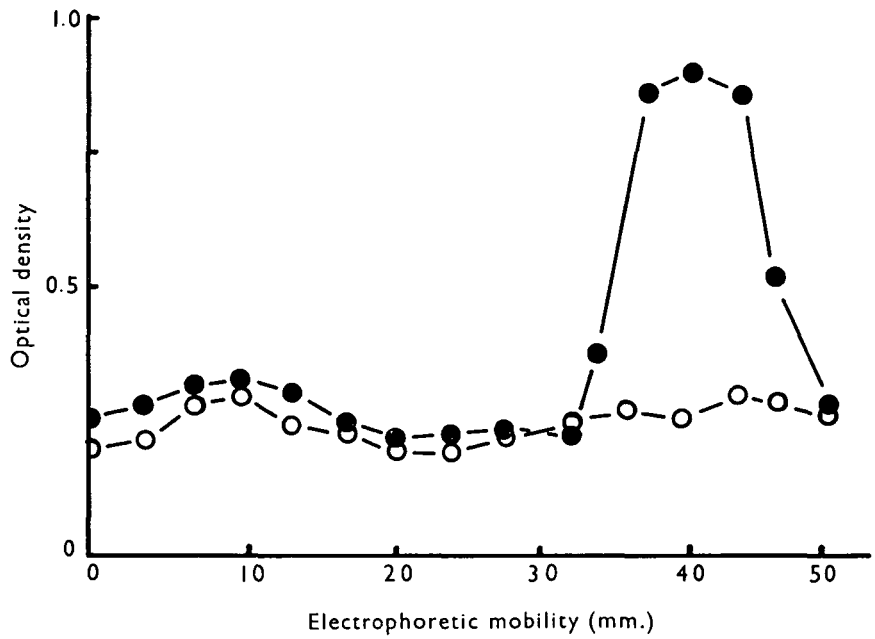
The fastest moving and most active band of esterase activity was the one which varied quantitatively in reaction according to the phenotype of the sheep. This was shown by using various plasma dilutions and naphthyl acetate as substrate; at the critical dilution, a band appeared for the plasma of higher activity but not for that of lower activity. In this way, Es^a/Es^a plasma could be distinguished from Es^a/Es^b and Es^a/Es^c plasma, which in turn were obviously different from Es^b/Es^b, Es^b/Es^c and Es^c/Es^c plasma.

The difference between Es^b/Es^b and Es^a/Es^b or Es^b/Es^c and between these and Es^a/Es^a, Es^a/Es^b and Es^c/Es^c was demonstrated by incubating gel strips (see Methods) with indophenyl acetate, and measuring the absorption of the blue colour by means of a spectrophotometer (Unicam SP 600, 625 mμ).

This method also gave a spectrum of activity for the whole gel, and a second band of esterase was located. The activity of this band in the hydrolysis of indophenyl acetate and also acetyl thiocholine, was found to be the same in all plasma samples. This separate slower-moving esterase probably contributes the low but measurable hydrolytic activity encountered in sheep without the appropriate allele.

The conclusion from the results obtained is that the esterase activity in sheep plasma is under quantitative genetic control, one allele governing the production of a reasonably well-defined amount of enzyme. Different alleles produce different esterases with respect to substrate affinities, but not with respect to electrophoretic mobility. One allele (Es^c) is responsible either for the production of a protein with no esterase activity, or no protein at all.

Fig. 1. The electrophoretic mobility and activity against indophenyl acetate of plasma esterases from sheep genotypes Es^a/Es^a (○) and Es^b/Es^b (●).



Conditions of electrophoresis: 30 mA., 150 V. for 60 min., in 1% agar-gel.
Conditions of hydrolysis: Indophenyl acetate (10^{-3}M), phosphate buffer, pH −8·0, 37°C. for 60 min.

This genetically controlled pattern of esterase activity in sheep is very similar to that found in man, where certain individuals are lacking in ability to hydrolyse succinyl choline. This lack may be partial or complete according to whether the individual is heterozygous or homozygous for the allele which produces atypical cholinesterase (Kalow, 1962). Three rates of atropine hydrolysis can be distinguished in rabbit sera, and this variation is also under genetic control (Sawin & Glick, 1943).

The tendency in sheep towards multiple allelism, for the control of esterase activity, resembles closely that of the pig (Augustinsson & Olsson, 1961), where individuals vary in their rate of phenyl acetate hydrolysis by plasma. However, on testing sixty samples of pig plasma, from blood obtained at a commercial slaughterhouse, it was found that none of the pigs had any plasma esterase activity with haloxon as substrate, nor was any hydrolysis of diethyl 4-nitrophenyl phosphate

(E 600, paraoxon) evident. This result indicates that the esterase studied by Augustinsson was a B-type esterase (Aldridge, 1953), in contrast to the A-esterase of sheep plasma.

Unfortunately, this study does not throw any light on the natural function of A-esterase, nor can any suggestion be offered as to the essential difference between the esters used which renders them so selective in their affinity for such electrophoretically similar proteins.

SUMMARY

1. The rate of hydrolysis by sheep plasma of some carboxylic and phosphate esters has been determined for a random flock, and for a flock previously selected for its ability to hydrolyse di-(2-chloroethyl) aryl phosphates.

2. A discontinuous variation in hydrolysis rate was found with all substrates tested and, using combinations of substrates, six types of plasma could be distinguished, each type having a different pattern of esterase activity.

3. The most useful substrates for distinguishing between phenotypes were 1-naphthyl acetate and 4-ethoxycarbonylcoumarin-7-yl acetate. Three rates of hydrolysis were possible for each of these esters, and the highest rate for one was invariably combined with the lowest rate for the other, although the converse did not apply.

4. To explain these results, and those of Lee (1964), it has been postulated that the quantitative production of esterase hydrolysing 1-naphthyl acetate is governed by the presence of an allele, termed Es^a, at a particular gene locus. Similarly, the production of esterase hydrolysing 4-ethoxycarbonylcoumarin-7-yl acetate is determined by allele Es^b, and where neither substrate is attacked the presence of a third allele, Es^c, is proposed.

5. The hydrolysis rates of haloxon, 1-naphthyl butyrate and 4-nitrophenyl butyrate varied in the same way as that of 1-naphthyl acetate, whereas the hydrolysis of indophenyl acetate followed the same pattern as that of 4-ethoxycarbonylcoumarin-7-yl acetate. The variation in hydrolysis rate of Coroxon could be explained by assuming that Es^a and Es^b are equal in this respect.

6. A mating experiment produced results which were in accordance with the genetic hypothesis, but were too few in number to provide confirmation.

7. The genetic marking of six types of sheep is possible, utilizing the variation in plasma A-esterase activity.

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