Genet. Res., Camb. (1967), **10**, *pp.* 135–142
With 2 text-figures
Printed in Great Britain

A serum esterase variation in *Microtus agrestis* (L.)

By M. A. RUSSELL* and R. SEMEONOFF†

Institute of Animal Genetics, Edinburgh

(*Received* 9 *March* 1967)

1. INTRODUCTION

Serum proteins can be separated into fractions by starch-gel electrophoresis (Smithies, 1955; Poulik, 1957). The process can be combined with histochemical staining methods to reveal the existence of enzymes which differ in electrophoretic mobility, within a group with a single catalytic specificity (Hunter & Markert, 1957). These methods applied to individuals from wild populations have revealed considerable variation affecting a large number of enzymes (Shaw, 1965), and have raised questions as to the adequacy of current population genetics theory (Lewontin & Hubby, 1966).

This report deals with the serum esterases of the field-vole *Microtus agrestis* (L.). Evidence has been found for two loci which control the synthesis of separate subunits of an esterase isozyme system. In the natural population under study, both loci appear to be polymorphic.

2. MATERIALS AND METHODS

The animals used were derived from a wild population of *M. agrestis* at Carron Valley, near Stirling, Scotland. This population will be described more fully elsewhere (Semeonoff & Robertson, in preparation) with respect to polymorphism of another esterase known as Es-1. The population was sampled at intervals by trapping live animals. After removal of blood samples in the laboratory, each animal was marked and released at precisely the place of capture. Marked individuals have frequently been recaptured several months later, indicating that the removal of a blood sample has no lasting effect. Other material came from a breeding, laboratory colony of *Microtus*, which is chiefly derived from animals from the Carron Valley study area.

The standard electrophoretic technique used throughout was developed by Semeonoff (Semeonoff & Robertson, in preparation). The gel is prepared using a 0·076 M Tris buffer (pH 8·6) and allowed to set overnight at room temperature. Twelve serum samples are introduced into the gel on filter paper inserts which are slotted vertically into slits cut in the gel. The gel is placed horizontally in an electrophoresis tank, the boxes of which contain Poulik's (1957) borate buffer (pH 8·9).

* When this work was carried out, a student for the degree of B.Sc. in the University of Edinburgh.

† Supported by the Nature Conservancy.

An ice pack placed on the gel minimizes heat denaturation of the serum enzymes during the run. A constant voltage circuit supplies a potential difference between the electrodes of 240 V., and the run takes about 2½ hours. After removal from the tank, the gel is sliced horizontally and routinely stained for esterases. Before staining one-half is preincubated in a 10^{-5} M solution of eserine in 0·15 M phosphate buffer (pH 6·5). Eserine inhibits certain esterases and is a useful aid to the identification of certain enzyme bands. The staining reaction depends on the coupling of a dye, Fast Blue B, to the hydrolysis product of the substrate, α-naphthyl-acetate, to give an insoluble pigment localized in the gel. The gel slices are stained in 10^{-3} M α-naphthyl acetate, 0·1% w/v Fast Blue B in 0·15 M phosphate buffer (pH 6·5). After 20 min. the solution is replaced with fresh, and staining continued for a further 20 min., when the reaction is terminated by addition of clearing solution, (5 parts methanol, 5 parts water, 1 part glacial acetic acid, v/v).

3. RESULTS AND DISCUSSION

Bands were identified on the three separate criteria of resistance to eserine inhibition (Augustinsson, 1959), colour of staining, and position with respect to bands not showing electrophoretic variations. Isozyme systems (Markert & Möller, 1959) were detected, in addition, by the patterns of association and exclusion which occurred between the component bands as a consequence of the structural properties which the enzymes of a system have in common. Variations in the properties of gel preparations were rendered unimportant by the routine inclusion of a well-characterized reference serum sample in each gel.

Reference to Fig. 1 demonstrates the high degree of complexity of the esterase phenotypes revealed by this method. Almost all the enzymes show some form of variation. For instance, the band labelled E_1 can be either present or absent in an individual and, if present, may be found in one of two alternative positions (Semeonoff & Robertson, in preparation). The four faintly staining E_3 bands migrating very close to the E_1 enzyme were selected for study. They are distinguished by a relative resistance to eserine inhibition and stain a grey colour. Preliminary population data indicated that the phenotypes are stable throughout the adult life of the animals and that the separate bands do not occur in random combinations in separate individuals. Figure 2 shows all the combinations actually found in single animals. There are a total of nine phenotypes out of a possible sixteen. Combinations which are absent are all groups of three bands, and certain groups of two, such as 'ad', and 'bc'. Other combinations of two—'ab', 'cd', 'bd'—are encountered. The simplest hypothesis which we could devise to explain this situation postulates that the four isozymes are variants of a basic dimer structure, and requires the existence of two loci which specify the sub-units of the dimer. The features of such a system would be:

1. The enzymes a, b, c and d are variant forms of a protein with a basically dimer structure consisting of two distinct polypeptide chains specified by genes at two loci.

2. Each of the two loci has two common alleles.
3. The allelic differences at both loci cause changes in the electrophoretic mobility of the enzymes they jointly specify.
4. The enzymes are formed by random association of the products of the two loci.

If the alleles are tentatively denoted C, C^1 and R, R^1 respectively, then in the heterozygote, C/C^1 R/R^1, all four forms of dimer can be formed giving the abcd

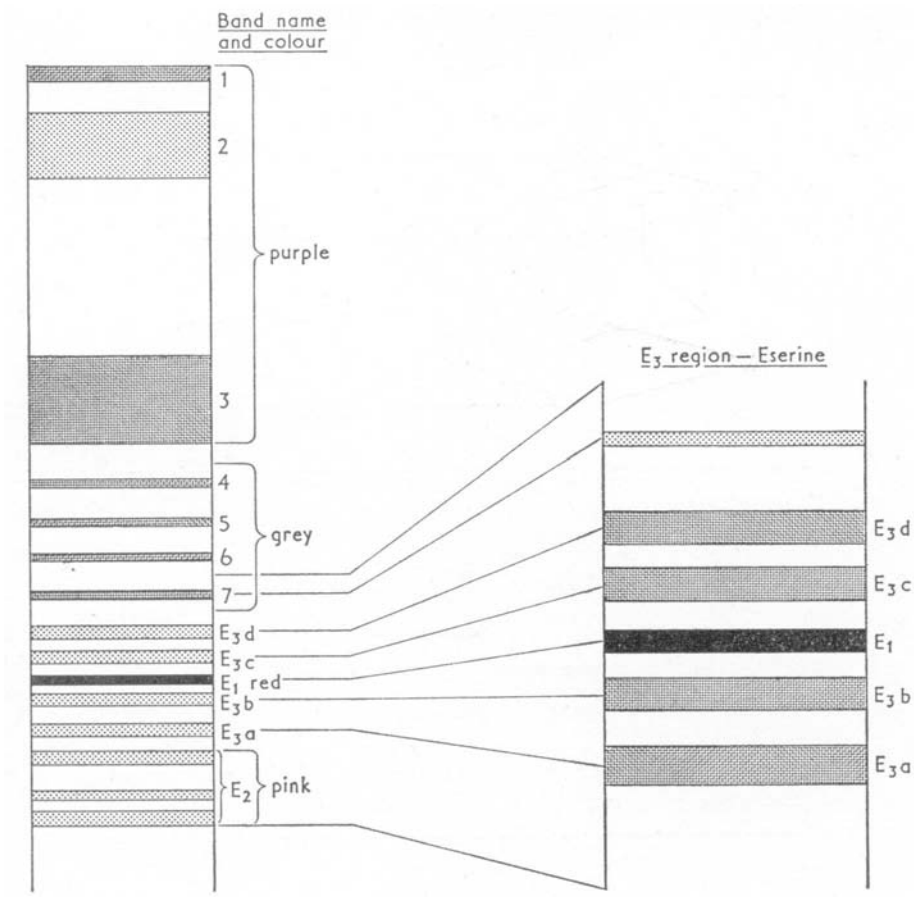
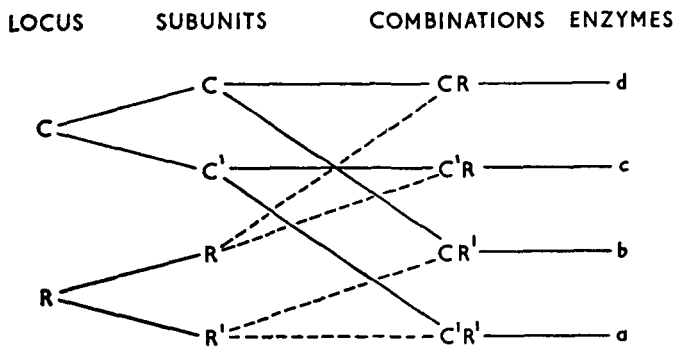


Figure 1.

phenotype. The generation of different phenotypes is explained diagrammatically in Fig. 2.

The hypothesis predicts that only the nine phenotypes illustrated in Fig. 2 will be found. The detection of even one 'forbidden' phenotype, such as any combination of three E_3 enzymes, would throw serious doubt on the validity of the hypothesis. Table 1 shows the results of a survey of 254 individuals to test this point. All nine expected phenotypes were observed, and no 'forbidden' phenotypes occurred. Most sera were examined more than once in different gels alongside well-characterized samples and were independently scored.

A further prediction of the theory is that the relative frequencies of various phenotypes should, in a random breeding population, approximate to those expected from the Hardy-Weinberg law. Maximum likelihood estimates of gene frequency were made for the three populations in the usual way, and expected genotype frequencies for both loci using the Hardy-Weinberg ratios: $p^2:2pq:q^2$. The significance of the deviations of the observed from the expected frequencies at each locus was tested using χ^2 with one degree of freedom. The χ^2 values given in Table 2 border on significance. Various explanations for these deviations were considered.



Genotypes	C'R' C'R'	C'R' C R'	C R' C R'	C'R' C'R	C'R' C R	C R' C R	C'R C'R	C'R C R	C R C R
Bands d					■	■		■	■
Bands c				■	■		■	■	
Bands b		■	■		■	■			
Bands a	■	■		■	■				
Phenotypes	a	ab	b	ac	abcd	bd	c	cd	d

Figure 2.

Inbreeding cannot explain them because it would cause an excess of homozygotes at both loci in a population sample. Selection could conceivably cause the observed differences if it operated in opposite directions in the wild and in the laboratory. Errors in scoring E_3 phenotypes could result in small erratic deviations from expectation.

To test this last possibility, the data were split into two groups on the basis of the E_1 phenotype. Because the E_1 enzyme migrates in our system between the E_3 b and c enzymes, it tends to obscure them. Animals lacking the E_1 band occur at a relatively high frequency in the Carron population. Table 3 shows a Hardy-Weinberg analysis applied as before, but using only E_1-negative animals. In this

https://doi.org/10.1017/S0016672300010879 Published online by Cambridge University Press

case none of the χ^2 values approaches significance, and the fit of the data to expectations is excellent. In addition, an analysis of collected data on repeat runs of serum samples, independently scored, showed that the probability of making an error in

Table 1. *The population data*

Phenotype	a	ab	b	ac	abcd	bd	c	cd	d	
Genotype	C^1R^1 C^1R^1	C^1R^1 CR^1	CR^1 CR^1	C^1R^1 C^1R	C^1R^1 CR	CR^1 CR	C^1R C^1R	C^1R CR	CR CR	
Population	Frequencies									Total
Laboratory	1	1	3	0	9	8	2	23	17	64
Carron (1)	0	2	3	0	4	4	9	23	27	72
Carron (2)	1	3	1	3	19	21	6	42	22	118
									Total	254

Table 2. *Deviations from Hardy-Weinberg equilibrium*

		Gene frequencies		Genotypic frequencies				
Population	Locus	E (p)	E (q)		Observed	Expected	$\chi^2_{(1)}$	Probability
Laboratory	C	0·684	0·316	CC	28	30	2·89	$>0·05<0·1$
				C^1C	33	28		
				C^1C^1	4	6		
	R	0·777	0·223	RR	42	34	4·80	$>0·02<0·05$
				R^1R	17	23		
				R^1R^1	6	3		
Carron (1)	C	0·674	0·326	CC	34	33	0·371	$>0·5$
				C^1C	29	32		
				C^1C^1	9	8		
	R	0·875	0·125	RR	59	55	data	—
				R^1R	8	16	too	
				R^1R^1	5	1	few	
Carron (2)	C	0·644	0·356	CC	44	49	3·94	$>0·02<0·05$
				C^1C	64	54		
				C^1C^1	10	15		
	R	0·775	0·225	RR	70	71	0·25	$>0·5$
				R^1R	43	41		
				R^1R^1	5	6		

scoring E_1-negative individuals is about one-half of that in E_1-positive ones. We also conducted a 'blind' scoring accuracy test, in which a number of replicate serum samples were run in a different order in a series of gels. The estimate of scoring accuracy thus obtained confirmed the conclusion that deviations from Hardy-Weinberg are caused by mis-scoring of E_1-positive sera.

The fact that three independent population samples show excellent agreement

https://doi.org/10.1017/S0016672300010879 Published online by Cambridge University Press

with Hardy-Weinberg expectations when scoring errors are minimized is good evidence for the validity of the proposed hypothesis. Other evidence is the absence of unexpected phenotypes in a total of 254 individuals examined. We therefore propose the symbols Es-3C and Es-3R for the loci, and to denote the alleles as Es-3C^a, controlling sub-unit C, and Es-3C^b controlling sub-unit C^1, and similarly, Es-3R^a and Es-3R^b controlling sub-units R and R^1 respectively. This scheme is in accordance with the notation used in recent work in this field.

This rather novel genetic situation can be exploited in a number of directions. First, the biochemical aspects of the E_3 system and its component enzymes might

Table 3. *Deviations from Hardy-Weinberg equilibrium. E_1 negative only*

		Gene frequencies		Genotypic frequencies				
Population	Locus	E (p)	E (q)		Observed	Expected	$\chi^2_{(1)}$	Probability
Laboratory	C	0·688	0·312	CC	13	15·15	3·07	$>0·05<0·1$
				C^1C	18	13·73	3·07	
				C^1C^1	1	3·12		
	R	0·781	0·219	RR	20	19·52	0·24	$>0·5$
				R^1R	10	10·95		
				R^1R^1	2	1·53		
Carron (1)	C	0·576	0·424	CC	10	10·95	0·45	$>0·5$
				C^1C	18	16·92		
				C^1C^1	5	5·93		
	R	0·909	0·091	RR	28	27·27	2·35	$>0·1$
				R^1R	4	5·46		
				R^1R^1	1	0·27		
Carron (2)	C	0·667	0·333	CC	18	18·69	0·21	$>0·5$
				C^1C	20	18·66		
				C^1C^1	4	4·65		
	R	0·678	0·322	RR	19	19·31	0·06	$>0·8$
				R^1R	19	19·34		
				R^1R^1	4	4·35		

be studied. Kaplan (1965) has presented evidence in the lactic dehydrogenase (LDH) isozyme system in mammals of a functional differentiation of the different isozymes. It has been suggested (Shaw, 1965) that a reason for the common occurrence of multimer enzymes is that this structure allows configurational changes of importance in the evolution of metabolic control systems. If some differences in the activity or specificities of the E_3 enzymes could be detected, the population genetics of the system would immediately become more significant. However, quite apart from the functional aspects, the genetics of the system is of considerable interest.

The wild population examined is polymorphic at both loci which jointly specify one enzyme. The situation in which the products of two structural genes co-operate in the synthesis of one enzyme has been described before, for instance xanthine dehydrogenase in *Drosophila melanogaster* (Smith, Ursprung & Wright,

1963). Polymorphism at the protein level is also widespread (Shaw, 1965). The two phenomena in combination, as in this system, are particularly suitable for examining the effects of joint selection on the two loci, if indeed selection is the force responsible for the maintenance of the high frequencies of the four alleles in the population. More data on other populations, and on the Carron population at future dates, will be required to establish this point. If selection is acting in this system, since the frequency of each phenotype is the joint product of the allelic frequencies at the two loci, the gene frequencies should show correlated changes, generating significant associations between alleles, if the effect were strong enough (Lewontin & White, 1960). The present data are not extensive enough to give more

Table 4. *Association of alleles in the three population samples*

		Grouped genotypic frequencies			
Population		CC	$C^1C+C^1C^1$	$\chi^2_{(1)}$	Probability
Laboratory	RR	10	10	1·94	$>0{\cdot}1$
	$R^1R+R^1R^1$	3	9		
Carron (1)	RR	10	18	2·57	$>0{\cdot}1$
	$R^1R+R^1R^1$	0	5		
Carron (2)	RR	4	15	6·71	$>0{\cdot}001<0{\cdot}01$
	$R^1R+R^1R^1$	14	9		

than a tentative indication on this possibility, but when a test for association between alleles was conducted on the March 1966 Carron sample, a highly significant ($\chi^2_{(1)}: p \leqslant 0{\cdot}01$) result was obtained. Table 4 shows this result. We feel therefore that this situation merits further study along both biochemical and genetical lines.

SUMMARY

1. An investigation of the serum esterases of the field-vole, *M. agrestis*, has been carried out by the method of starch-gel electrophoresis.
2. A total of 254 animals, mainly from Carron Valley, near Stirling, have been examined.
3. Evidence is presented for a system of four enzymes controlled by two genetic loci, which it is proposed to call Es-3C and Es-3R.
4. Each locus specifies one polypeptide sub-unit of the E_3 enzyme, which is a dimer.
5. The two loci each have two alleles present at high frequencies in the population examined.

Our sincere thanks are due to Dr F. W. Robertson for his advice and disucssion during the course of the work and in the presentation of the manuscript, and to Mr E. D. Roberts who prepared the diagrams.

REFERENCES

Augustinsson, K. B. (1959). Electrophoresis studies on blood plasma esterases. *Acta chem. scand.* **13**, 571.

Hunter, R. L. & Markert, C. L. (1957). Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science, N.Y.* **125**, 1294.

Kaplan, N. O. (1965). Lactic dehydrogenase—structure and function. *Brookhaven Symp. Biol.* **17**, 131.

Lewontin, R. C. & Hubby, J. L. (1966). A molecular approach to the study of genetic heterozygosity in natural populations. II. *Genetics*, **54**, 595.

Lewontin, R. C. & White, M. J. D. (1960). Interaction between inversion polymorphism of two chromosome pairs in *Moraba scurra*. *Evolution, Lancaster, Pa.* **14**, 116.

Markert, C. L. & Möller, F. (1959). Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. *Proc. natn. Acad. Sci. U.S.A.* **45**, 753.

Poulik, M. D. (1957). Starch gel electrophoresis in a discontinuous system of buffers. *Nature, Lond.*, **180**, 1477.

Semeonoff, R. & Robertson, F. W. (in preparation).

Shaw, C. R. (1965). The use of genetic variation in the analysis of isozyme structure. *Brookhaven Symp. Biol.* **17**, 117.

Smith, K. D., Ursprung, H. & Wright, T. R. F. (1963). Xanthine dehydrogenase in *Drosophila*: detection of isozymes. *Science, N.Y.* **142**, 226.

Smithies, O. (1955). Zone electrophoresis in starch gel. *Biochem. J.* **61**, 629.