GENETIC VARIATION IN RED CELL ESTERASES OF RABBITS[1]

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INTEREST in searching for genetic variation in esterases was stimulated by the work of MARKERT and HUNTER (1959) who observed that electrophoretic differences between the esterases of various tissues in mice can be readily demonstrated in starch gels by applying appropriate histochemical staining techniques. Since then, genetic variation in esterases has been reported in studies of maize endosperm and seedlings (SCHWARTZ 1960), Tetrahymena cultures (ALLEN 1960), the serum of mice (POPP 1961), man (HARRIS, HOPKINSON and ROBSON 1962) and horses (OKI, OLIVER and FUNNELL 1964), homogenates of fruit flies (WRIGHT 1963; JOHNSON 1964) and house flies (VELTHIUS and VAN ASPEREN 1963) and in red cells of man (TASHIAN and SHAW 1962) and cattle (SARTORE, GRUNDER and STORMONT 1965). What was at first believed to be a second genetic system of human red cell esterases (SHAW, SYNER and TASHIAN 1962) was later classified as a system involving carbonic anhydrases (TASHIAN and SHAW 1962; TASHIAN, PLATO and SHOWS 1963). Additional references on this subject may be found in a review by SHAW (1965). This report is concerned with genetic variation in red cell esterases of domestic rabbits (*Oryctolagus cuniculus*).

MATERIALS AND METHODS

Sources of samples: Blood samples, drawn from the marginal ear vein either into Alsever's solution or citrate solution (2% sodium citrate and 0.5% sodium chloride), were obtained from rabbits (New Zealand Whites and Californians) in the breeding populations maintained by the U.S. Department of Agriculture in Fontana, California, and from rabbits of mixed breeding (largely New Zealand Whites) maintained as a nonbreeding population in our Laboratory.

Preparation of hemolysates: Erythrocytes from individual rabbits were washed three times in 10 volumes of saline solution (0.90% NaCl) per washing. The washed, packed cells were lysed in 2 volumes of distilled water and stored at —18° C when not in use.

Electrophoresis: Horizontal starch-gel electrophoresis was performed on the hemolysates. The best resolution of esterase bands or zones was obtained by using an electrolyte buffer of 0.0286 M LiOH and 0.191 M boric acid (after GAHNE 1963) and a gel buffer made by mixing 40 ml of electrolyte buffer with 210 ml of a solution containing 0.0715 M Tris(hydroxymethyl) aminomethane and 0.0228 M monohydrated citric acid. Each gel consisted of 250 ml of the gel buffer plus 32g of hydrolyzed starch (Connaught Medical Research Laboratories, Toronto) and the gels were prepared in the manner described by KRISTJANSSON (1960, 1963), the gel-frame measuring 130 × 215 × 6 mm.

Hemolysates, absorbed onto 9 × 6 mm paper wicks (Beckman Instruments Inc. paper No.

319329), were inserted 3 cm from the cathodic end at right angles to the long axis. Usually ten samples were run per gel. Electrophoresis was begun by applying 165 volts for 30 minutes at which time the filter paper inserts were removed, ice packs were placed on the gel and the voltage was increased to 350. Electrophoresis was continued at 350 volts until the leading band of a sample of writing ink (Parker Co., Permanent Blue-Black), inserted as a marker, had migrated 10 to 11 cm from the point of insertion. (The total time of electrophoresis was approximately 11 hours.) The gels were then sliced horizontally into halves or thirds depending upon the number of staining operations and inhibition tests to be performed. Further details of procedure can be found in a thesis by GRUNDER (1966).

Staining: For the routine identification of esterase phenotypes, one slice of the gel was immersed in a freshly prepared staining solution containing 94 ml H_2O, 4 m lof 0.2 M phosphate buffer ph 6.8, 2 ml of a 1% solution of α-naphthyl butyrate and 100 mg of diazonium dye coupler (after MARKERT and HUNTER 1959). Either of the salts, Blue RR or Fast Blue BB was effective as coupler. The bands of esterase activity were well developed by 2 hours. The stained gels were rinsed in water and fixed in a 5:5:1 solution of methanol, water and glacial acetic acid to make them more amenable for handling and storage.

Identification of the kinds of esterases: Attempts were made to identify the specific kinds of esterases using the criteria set forth by AUGUSTINSSON (1958, 1961). The method is based largely on the ability of certain substances to inhibit the reactions between esterases and their various substrates. In this study 10^{-4} M di-isopropyl fluorophosphate (DFP), 10^{-4} M ethylenediamine-tetraacetic acid (EDTA) and 0.5×10^{-4} M eserine sulfate were used as inhibitors. Acetoazol-amide, a specific inhibitor of carbonic anhydrase, was used at 10^{-5} M. The inhibition tests were performed in a manner similar to those described by ALLEN (1960) and WRIGHT (1963). One of the slices of gel was placed in 50 ml of a given inhibition solution for 1 hour. It was then immersed overnight in a second solution (50 ml) containing that same concentration of inhibitor along with substrate (α-naphthyl butyrate), phosphate buffer and dye coupler in amounts used for routine staining. A second slice of the gel was stained in the routine manner already described.

Also, in connection with attempts to identify specific kinds of esterases, α-naphthyl acetate, α-naphthyl propionate, α-naphthyl caprylate, β-naphthyl acetate and β-carbonapthoxy choline iodide, in addition to α-naphthyl butyrate, were used as substrates but only α-naphthyl butyrate was routinely employed as substrate in the inhibition tests.

RESULTS

Esterase phenotypes: On all gels there were three disjunct regions of esterase activity which, for purposes of description, are designated I, II and III in order of their proximity to the anodic end of the gels (Figure 1).

Samples from all rabbits showed a single band or zone of esterase in Region I, and there was no variation between rabbits with respect to the appearance and electrophoretic position of that band.

Similarly, all samples showed a single band of esterase in region II. However, after prolonged storage of the hemolysates at $-18°C$ this band became more diffuse, thereby suggesting differences between rabbits when the samples being run varied in length of storage. There were, however, no real differences between rabbits with respect to that esterase when the lysates were of like age.

There were five zones of esterase activity in Region III. The five bands are numbered 1 through 5 (Figure 1) in decreasing order of mobility. Bands 1 and 2 were usually separated by a distance of approximately 6 mm. Bands 2, 3 and 4 were rather evenly spaced and were separated from one another by a distance
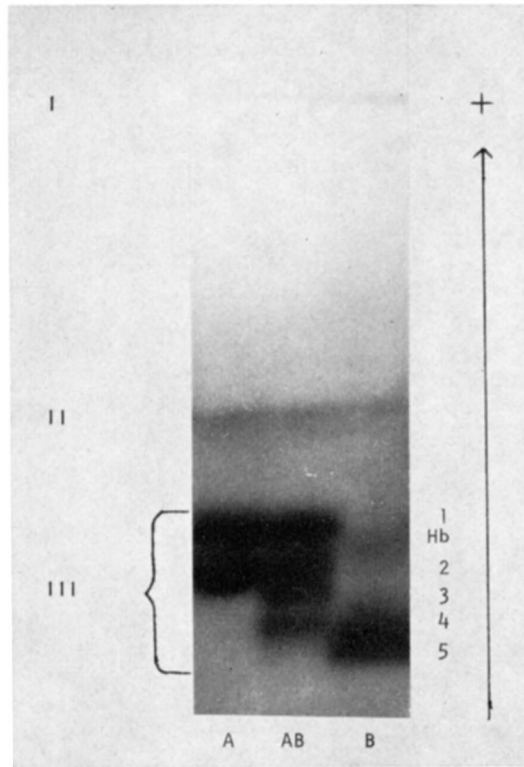
FIGURE 1.—A portion of a stained gel showing the appearance of the esterase bands in phenotypes A, B and AB. Areas of esterase activity occurred in three regions designated I, II and III in the figure. Phenotypic variation was observed only in Region III. A hemoglobin band (labelled Hb), clearly discernable only in phenotype B, lies between bands 1 and 2. Phenotype A is characterized by bands 1 and 2, phenotype B by bands 4 and 5, and phenotype AB by bands 1, 2, 3, 4 and 5.

of approximately 2.5 mm. The distance between bands 4 and 5 was somewhat less, and in most gels it was difficult to discern a distinct line of demarcation between them.

With respect to the five zones of esterase in Region III, there were three clearly distinguishable phenotypes. These were named A, B and AB on the premise that the three types might be controlled by a single pair of codominant alleles. These phenotypes are shown in Figure 1. Phenotype A is characterized by bands 1 and 2, phenotype B by bands 4 and 5, and phenotype AB (the proposed heterozygote) by all five bands. Particularly diagnostic of phenotype AB was the presence of band 3, a band which was never seen in either of the proposed homozygotes nor in runs performed on artificial compounds made by pooling hemolysates from type A and type B rabbits.

Bands 1 and 2 in phenotype A appeared to represent equal esterase activity. Usually band 1 appeared somewhat wider but slightly less intense than band 2.

In contrast, band 1 in phenotype AB stained to about the same degree of intensity as bands 1 and 2 in phenotype A, but band 2 in phenotype AB always stained less intensely than 1.

Bands 4 and 5 in phenotype B appeared to stain with about equal intensity. In contrast, band 4 in phenotype AB stained to almost the same degree of intensity as its counterpart in phenotype B, but band 5 was always much less intensely stained than its counterpart in phenotype B. Indeed, in most gels band 5 in phenotype AB was so faintly stained that it was difficult to discern. For example, in Figure 1 band 5 in phenotype AB does not show in the photograph but it was faintly present on the gel. As ascertained by performing repeated runs, there were no animals of phenotype AB which lacked band 5. When the amount of starch was reduced from 32 to 27 g per gel, band 5 in phenotype AB became much more pronounced and was clearly separated from band 4. But, at the same time, band 4 in phenotype B became much less distinct and was represented only by a smear or beard on the anodic side of band 5. Also, bands 1 and 2 in phenotypes A and AB became more closely spaced.

Band 3, limited to phenotype AB, was always clearly represented and stained to about the same degree of intensity as bands 2 and 4 in this phenotype.

By comparing hemolysates which had been stored at −18°C for 48 hours (or less) with replicates stored for several weeks, it became evident that the intensity of staining increased somewhat with storage. Also, a very faint band, located just ahead of band 2, appeared in aged hemolysates from type A rabbits but was particularly difficult to discern because of the presence of hemoglobin which migrated in the region between bands 1 and 2 (see Figure 1). This extra band, occasionally observed in hemolysates from rabbits of type AB, was never observed in rabbits of phenotype B. Occasionally band 1 of phenotypes A and AB appeared as two closely spaced bands but this was largely an effect of using aged lysates.

Runs were performed on fresh or fresh-frozen serum samples, using the standard procedure, to determine whether the esterase bands observed in hemolysates would also be evident in sera. Bands which appeared to be homologous to those of Regions I and III of the lysates were observed in the runs performed on serum samples. Other esterase bands were also observed in the sera. Routine diagnosis of the three phenotypes was, however, more reliable using hemolysates.

Classification of the kinds of esterases: In Table 1 are summarized the results of tests with the various inhibitors and substrates. As shown in that table, the esterase in Region I was not inhibited by any of the inhibitors (i.e., DFP, EDTA and eserine sulfate) and it readily hydrolyzed all substrates except β-carbonapthoxy choline iodide. Because the esterase of Region I was insensitive to DFP and eserine sulfate and because it migrated the farthest toward the anode, it is tentatively classified as an arylesterase. Although AUGUSTINSSON (1961) pointed out that most arylesterases are sensitive to EDTA, the esterase of Region I could be an arylesterase resistant to EDTA.

The esterase in Region II was not inhibited either by EDTA or eserine sulfate. It was, however, partially inhibited by DFP. (When DFP was used at 10^{-3} M rather than 10^{-4}, the activity was completely inhibited.) The esterase in Region

TABLE 1

Intra-regional comparison of the staining intensity of bands representing esterase activity of red cell lysates using various substrates and inhibitors

Substrate	Inhibitor	Staining intensity* of Regions		
		I	II	III
α-naphthyl butyrate	....	+	+	+
α-naphthyl butyrate	EDTA	+	+	+
α-naphthyl butyrate	Eserine Sulfate	+	+	+
α-naphthyl butyrate	DFP	+	tr/±	—
α-naphthyl butyrate	Acetoazolamide	+	+	+
α-naphthyl acetate	....	+	+	+
α-naphthyl propionate	....	+	+	+
α-naphthyl caprylate	....	±	—	—
β-naphthyl acetate	....	+	—	tr
β-carbonapthoxy choline iodide	....	tr	—	—

* + (normal staining intensity); ± (light staining); tr (trace); — (no staining).

II readily hydrolyzed α-naphthyl acetate, α-naphthyl propionate and α-naphthyl butyrate but showed no activity with the remaining three substrates. These results suggest tentative classification of the esterase of Region II as an aliesterase.

The esterases in Region III, irrespective of phenotypes, behaved alike in all tests. The activity of those esterases was not inhibited either by EDTA or eserine sulfate but was completely inhibited by DFP. And, as in the case of the esterase of Region II, those in Region III readily hydrolyzed α-naphthyl acetate, α-naphthyl propionate and α-naphthyl butyrate but showed no activity when α-naphthyl caprylate and β-carbonapthoxy choline iodide were used as substrates. However, in contrast with the esterase of Region II, those of Region III showed a trace of activity when β-naphthyl acetate was used as substrate. The esterases of Region III have the main requirements of an aliesterase, i.e., sensitivity to DFP and insensitivity to eserine sulfate and EDTA.

The observation that acetoazolamide, used at 10^{-5} M, failed to inhibit any of the zones of enzymatic activity indicates that the enzymatic sites which are responsible for the esterase activity have no carbonic anhydrase activity. This is in contrast with the results of SHAW et al. (1962) in their studies of human red cell esterases. They observed that certain of the human enzymes were inhibited by acetoazolamide.

The uniformity of response of all the zones of esterase in Region III when tested with the various inhibitors and substrates provides evidence that the three phenotypes A, B and AB are multiple forms (isozymes) of the same enzyme. Additional evidence to that effect now follows.

Family data: The distribution of the three phenotypes A, B and AB in 429 offspring from the six possible kinds of matings is shown in Table 2. With one exception, namely, the occurrence of an offspring of phenotype A from a B × AB mating, the results are in agreement with expectation based on a single pair of codominant autosomal alleles, Es^A and Es^B.

TABLE 2

Distribution of esterase phenotypes in progeny from the various matings

		No. of progeny of phenotypes		
Matings	No. of litters	A	B	AB
A × A	0	0	0	0
A × B	2	0	0	15
A × AB	1	2	0	6
B × B	13	0	64	0
B × AB	30	1*	87	93
AB × AB	26	42	43	76

* Exception to the genetic theory (see text).

While care was taken to exclude from the family studies any litters about which there was any question regarding legitimacy of the offspring, the occurrence of one offspring of phenotype A from a B × AB mating raised this question anew. On checking this with the management of the Fontana Station it was found that one litter of rabbits containing a fostered rabbit had been accidently included among those sampled in the family studies. As it turned out, this was the same litter which contained the exceptional offspring. Because there was no record to show which of the seven rabbits in that litter was the fostered rabbit, there was no absolute proof that the exceptional rabbit was the fostered rabbit. Therefore, we felt obliged to call attention to these events and let the readers draw their own conclusions.

Population data: Estimates of allelic frequencies for three populations are presented in Table 3. Only adults and only one rabbit (randomly selected) of each sib family was included in the analyses of the New Zealand White and Californian populations at the Fontana Station. In these two populations the frequency of the Es^A allele was 0.32 and 0.06 respectively. In the heterogeneous laboratory stock, largely of New Zealand White ancestry, the frequency of allele Es^A was 0.34, almost the same as that of the New Zealand White population at the Fontana Station.

The expected phenotypic frequencies for the Serology Laboratory rabbits are compared with the observed in Table 4. The close agreement between observed and expected numbers supports the explanation based on a pair of alleles.

TABLE 3

Estimates of the frequency of alleles in three adult populations

		Frequency of alleles	
Populations	Adults	Es^A	Es^B
New Zealand White	14	0.32	0.68
Californian	9	0.06	0.94
Serology Laboratory	89	0.34	0.66

TABLE 4

Comparison of observed phenotypic frequencies of red cell esterases with expected frequencies in a sample of 89 Serology Laboratory rabbits

	Phenotypes		
	A	B	AB
Observed	11	39	39
Expected	10	40	39

DISCUSSION

If it be accepted that the multiple bands of esterase in Region III of the gels are controlled by alleles, there is still the problem of accounting for the production of two enzyme bands by one allele ($Es^A \rightarrow$ bands 1 and 2; $Es^B \rightarrow$ bands 4 and 5), and, of explaining how the two alleles in heterozygous combination can produce a "hybrid" zone (band number 3). An example of a protein exhibiting similar phenomena is human haptoglobin in which allele Hp^2 controls multiple bands of haptoglobin while the heterozygote ($Hp^1 Hp^2$) exhibits numerous zones of "hybrid" haptoglobin (SMITHIES 1959; CONNELL, DIXON and SMITHIES 1962). The examples of "hybrid" enzymes are now numerous. SHAW (1965) lists 19 examples, including the present example. With respect to these red cell esterases there is presently no information on their physical nature to aid in characterization of the various bands. Furthermore, it is unlikely that these esterases would retain any enzymatic activity following, for example, reductive cleavage, a treatment used to elucidate haptoglobin subunit structure. Nevertheless, the authors offer the following model as a working hypothesis.

Assume that allele Es^A acts to form a monomer which is represented by band 1 in the gels and that some of the protein of this monomer polymerizes to form a stable dimer (designated 1-1) which is represented by band 2 in the gels. Likewise, assume that allele Es^B acts to form a monomer (band 4) and that some of this protein polymerizes to form a stable dimer (4-4) identifiable as band 5 in the gels. If so, it would be expected that a third stable dimer (1-4) would be formed in the heterozygote, thereby accounting for zone number 3, the so-called "hybrid" band.

While there are certain quantitative aspects of the appearance of the esterase zones in the heterozygote which would suggest that some of the esterase of zones 1 and 4 is used in the formation of that in zone 3, one would have to suppose that the formation of dimer 1-4 (band 3) takes place largely at the expense of dimer 4-4 (band 5) in order to account for the marked reduction of esterase activity in zone 5 of the heterozygote. Furthermore, and judging wholly on the basis of staining intensity of the various zones, it would appear that allele Es^A manufactures more esterase than allele Es^B. However, it should be kept in mind that staining capacity of the various zones is a measure of esterase activity rather than quantity of esterase, and that there could be little relation between the amount

of α-naphthyl butyrate hydrolyzed and the actual concentration of proposed monomer or dimer.

Another way to account for multiple bands controlled by individual alleles is through the association of their macromolecular products with components of the starch or gel buffer. PARKER and BEARN (1963) have shown that a switch from a Tris-citrate or phosphate-citrate buffer to a boric acid-sodium hydroxide buffer allows a major amido-black stained component of conalbumin to produce two additional slower migrating components. These authors suggested that complexes of boric acid plus conalbumin are responsible for the new bands. It is conceivable that small uncharged constituents in the buffer solutions bind with the allelic products to produce multiple bands, a possibility suggested by the observations of CANN and GOAD (1965). Such a type of binding could be responsible for the altered migration pattern of bands observed when, in the present study, only 27 instead of 32 g of starch were used to make the gel.

SUMMARY

Three regions of esterase activity designated I, II and III were evident when lysates of rabbit red cells were subjected to electrophoresis in starch gels and stained with appropriate histochemical stains. There was no variation in the singlebanded esterases of Regions I and II. Three multibanded phenotypes were observed in Region III. They are designated A, B and AB, and appear to be inherited as if controlled by a single pair of autosomal alleles Es^A and Es^B. The heterozygote exhibits all bands of the two homozygotes and, in addition, a "hybrid" band. The esterases in Regions II and III appear to be aliesterases while that in Region I is tentatively classified as an arylesterase.

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