GENETIC CONTROL OF ERYTHROCYTIC ESTERASE FORMS IN MUS MUSCULUS

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STARCH or agar gel electrophoresis coupled with azo dye staining methods has permitted the detection of genetically determined variants of several forms of nonspecific carboxylic esterases in a protozoan (Allen 1961), maize (Schwartz 1960, 1964), Drosophila (Wright 1963; Wright and MacIntyre 1963), and house flies (Velthuis and van Asperen 1963). In mammals, inherited electrophoretic variants of esterases have been reported from the serum (Popp and Popp 1962; Petras 1963) and kidney (Ruddle and Roderick 1965) of the house mouse (Mus musculus), and from red cell lysates of the deer mouse (Peromyscus maniculatus) (Randerson 1964) and of man (Tashian 1965). The present report describes the genetic control, and preliminary chemical characterization, of some electrophoretic forms of nonspecific esterases in hemolysates of the house mouse.

MATERIALS AND METHODS

Animals: Eleven inbred strains [A/He (A), AT, BALB/c (C), CBA/J, C3H/He, C57BL/6, C57BL/10 (B), C57BL/10-1u, C57BR/cd, DBA/2J and SEC/Re] and three partially inbred stocks (HL-ZRDCT-An, LL-ZRDCT-An and F1x/Dil) were surveyed for erythrocytic esterase types. Reciprocal crosses between (a) A/He and BALB/c (hereafter referred to as ACF1), (b) A/H and C57BL/10 (ABF1) and (c) BALB/c and C57BL/10 (CBF1) were performed to obtain F1 progeny for genetic study of the red cell esterase phenotypes. F1 hybrids were intercrossed and also backcrossed to their respective parental lines. Offspring were weaned at about four weeks and individually identified.

Preparation and electrophoresis of hemolysates: Since various qualitative and quantitative changes in numerous proteins have been shown to occur during the first few weeks after birth (Shreffler 1960; Popp and Popp 1962; Petras 1963), mice were not bled until they had reached at least eight weeks of age. Mice were bled by rotation of a 1.6 to 1.8" glass capillary tube inserted into the suborbital canthal sinus of the eye. The blood samples were numerically coded to avoid any possible source of bias in classification of animals according to erythrocytic esterase type. Thus, identification as to strain or type of cross was not recorded until after the hemolysate had been typed for esterase pattern. The coded samples were either tested fresh, or frozen and stored at -20°C until used.

The preparation of hemolysates, and the starch gel electrophoresis and subsequent azo dye staining methods were carried out as previously described (Tashian and Shaw 1962). Hemolysates were subjected to vertical starch gel electrophoresis employing a gel buffer solution of 0.02M boric acid adjusted to pH 8.6 with NaOH and a bridge buffer of 0.3M boric acid and 0.05M NaCl at pH 8.0. Satisfactory resolution was achieved at a gradient potential of about 6 v/cm.

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for 14 to 16 hours at 3° to 5°C. The zones of esterase activity on the starch gel were usually detected by using α-naphthyl acetate as the substrate; however, the following naphthol esters were also used to determine substrate affinities of the various esterase forms: α-naphthyl propionate, α-naphthyl butyrate, β-naphthyl acetate, β-naphthyl butyrate, naphthol AS-D acetate and indoxyl acetate.

Inhibition and activation studies: For most of these studies the gels were incubated at room temperature in the presence of the inhibitor (or activator) and Tris buffer for about 15 to 30 minutes prior to the addition of the substrate-dye mixture. Controls from the same starch gel were incubated in the Tris buffer without inhibitor (or activator) for the same amount of time and then stained with the standard staining solution. In order to determine the effect of alkylating agents, the hemolysate samples were incubated with the reagent at room temperature for 30 minutes before electrophoresis of the lysate. The inhibitors and activators used for further characterization of the red cell esterases were diisopropyl fluorophosphate (DFP), eserine sulfate, ethylenediamine tetraacetate (EDTA), iodoacetamide, p-chloromercuribenzoic acid (CMB), acetazolamide, 1,10 phenanthroline and 1:5-bis-(4allyl dimethylammoniumphenyl) pentan-3-one dibromide (= B.W. 284C51 dibromide).

Detection of other inherited traits. Coat color, hemoglobin and histocompatibility-2 (H-2) genotypes were ascertained to permit linkage studies of crosses segregating for these traits. The genotypes of all these traits can be determined for most of the mice directly from the phenotype observed. Coat color was judged by gross visualization and hemoglobin type by the method of starch gel electrophoresis (Russell and Gerald 1958). The red cell H-2 phenotype was ascertained by Dr. Donald C. Shreffler according to the polyvinyl pyrrolidinone (P.V.P.) method of hemagglutination (Stimpfling 1961). Each blood sample was serotyped with one to six different reagents at typing dilutions of 1/40 or 1/60. H-2 typing was completed within two days after collection of the blood.

RESULTS

Esterase zymograms from mouse hemolysates: Up to 11 anodal sites of esterase activity ("bands") were resolved from mouse red cell lysates under the present conditions (Figure 1). Cathodally migrating esterases were not observed. These enzymes have been designated Ee-1a (erythrocytic esterase-1a), Ee-1b, Ee-3, Ee-4, Ee-5, Ee-6, Ee-2a, Ee-7, Ee-8, Ee-9 and Ee-10 in order of decreasing electonegativity.

A characteristic esterase pattern was obtained for individuals of both sexes of any given mouse stock. However, variations with respect to the Ee-1 and Ee-2 esterases were observed among the mice examined. A survey of 11 inbred strains and three partially inbred stocks showed that hemolysates of some stocks constantly possessed the fast migrating form of Ee-1 esterase (Ee-1a), whereas the others exhibited the slow Ee-1b form (Table 1). These mouse stocks also differed in the presence (Ee-2a) and absence (Ee-2o) of esterase Ee-2a. The Ee-1 and Ee-2 esterase phenotypes of three inbred strains are shown in Figure 2.

Properties of the Ee-1 and Ee-2 esterases: The substrate used for esterase type classification, inhibition, activation and thermostability studies was α-naphthyl acetate. Various other α-naphthol, β-naphthol and indoxyl esters were employed to determine the substrate affinities of each esterase form as determined by staining intensity with the particular ester. Esterases Ee-1a and Ee-1b hydrolyzed the esters of α-naphthol in the order: acetate > propionate > butyrate; whereas esterase E-2a showed the order: propionate > acetate > butyrate. On the basis of affinity for α-naphthol esters, esterases Ee-1a and Ee-1b are acetylesterases
and Ee-2a is a propionylesterase. These three esterases also reacted with 
\[ \beta \]-naphthyl acetate, \( \beta \)-naphthyl butyrate and naphthol AS-D acetate. Esterases 
Ee-1a and Ee-1b showed greater reactivity with \( \alpha \)-naphthyl acetate than with the 
three \( \beta \)-naphthol esters tested; however, esterase Ee-2a preferentially hydrolyzed 
naphthol AS-D acetate over \( \alpha \)-naphthyl acetate, attacked \( \alpha \)-naphthyl acetate in 
preference to \( \beta \)-naphthyl butyrate and reacted equally well with the acetate 
esters of \( \alpha \) - and \( \beta \)-naphthol. Still another difference between these esterases was 
the ability of esterase Ee-2a to cleave indoxyl acetate. Ee-1a and Ee-1b failed to 
show activity when indoxyl acetate was used as substrate.

Esterases Ee-1a and Ee-1b were sensitive to DFP \((5 \times 10^{-3} \text{M})\) and Ee-2a was 
inhibited by eserine \((10^{-3} \text{M})\). Both the Ee-1a and Ee-1b forms were affected by 
DFP to the same degree and apparently differ only in their electrophoretic

### Table 1

**Ee-1 and Ee-2 erythrocytic esterase phenotypes among stocks of Mus musculus**

<table>
<thead>
<tr>
<th>Stock</th>
<th>Erythrocytic esterase type</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6, C57BL/10, C57BL/10-\text{Lu} and C57BR/cd</td>
<td>Ee-1a, Ee-2a</td>
</tr>
<tr>
<td>AT, BALB/c and SEC/Re</td>
<td>Ee-1a, Ee-2a</td>
</tr>
<tr>
<td>A/He, CBA/J, C3H/He, DBA/2J, F1x/Dil,</td>
<td>Ee-1b, Ee-2a</td>
</tr>
<tr>
<td>HL-ZRDCT-An and LL-ZRDCT-An</td>
<td>Ee-1b, Ee-2a</td>
</tr>
</tbody>
</table>
mobility. The activity of these three esterases was enhanced by the addition of EDTA ($10^{-3}$M) to the staining solution. Iodoacetamide ($3 \times 10^{-3}$M), 1,10 phenanthroline ($10^{-3}$M) and B.W. 284C51j dibromide ($10^{-3}$M) were without effect on these esterase forms.

Treatment of hemolysates with CMB ($8 \times 10^{-4}$M) appeared to cause esterase Ee-2a to migrate anodally to the area between Ee-1b and the site occupied by Ee-2a. Under similar treatment with CMB, an anodal electrophoretic shift was demonstrated for human red-cell carbonic anhydrase I (TASHIAN 1964) and A$_3$ esterase (TASHIAN, unpublished). It is also possible, however, that esterase Ee-2a was inhibited and another esterase form migrating between esterases Ee-1b and Ee-2a was activated by this compound in the present study.

Acetazolamide ($2.0 \times 10^{-4}$M), an enzyme inhibitor that acts specifically on carbonic anhydrase, failed to inhibit these esterase forms, thus indicating the absence of a carbonic anhydrase with esterase activity (TASHIAN, DOUGLAS and Yu 1964).

Heating hemolysates for 10 minutes at 55°C resulted in a loss of esterase Ee-2a activity. Esterases Ee-1a and Ee-1b remained stable after such treatment.

*Genetic control of the Ee-1 esterases:* The Ee-1 esterase phenotypes of A/He, BALB/c and C57BL/10 parental mice and their offspring are shown in Table 2. Certain F$_1$ hybrids contain both Ee-1 esterases and will be referred to as Ee-1 (a + b). Sex-linkage appears to be excluded by the observations that the observed phenotypes are similar for both sexes of any given cross and reciprocal crosses produce similar esterase types.
The phenotypic distribution of the Ee-1 esterase types among intercross (F₂) and backcross (BC) animals is presented in Table 3. The data on males and females from F₁ and BC matings have been pooled for Table 3 because no significant differences have been noted between the sexes as judged by χ² analysis. The results of Tables 2 and 3 are consistent with the simple genetic hypothesis that codominant autosomal alleles at a single locus specify the Ee-1a and Ee-1b esterases. It is noted that only expected phenotypes are observed. Further, good agreement between the observed values and theoretically possible values is seen to exist in all crosses with the χ² and probability values revealing no significant deviations.

In accordance with the rules set forth by The Committee on Standardized Genetic Nomenclature of Mice (1963), it is proposed that esterases Ee-1a and Ee-1b are determined by one locus, Ee-1 (erythrocytic esterase locus number one), with the autosomal codominant allele Ee-a responsible for the production of a form of Ee-1 that migrates faster than that produced by the other allele Ee-b. In the homozygous condition alleles Ee-a and Ee-b produce esterase forms shown by the single Ee-1a and Ee-1b esterases respectively, with characteristic
electrophoretic mobilities. Heterozygous mice \((\text{Ee-1}^a/\text{Ee-1}^b)\) display both the fast Ee-1a and slow Ee-1b esterases.

**Genetic control of the Ee-2 esterase:** The Ee-2 esterase phenotypes of the three parental mouse strains and their offspring are given in Table 4. F₁ hybrids possessing Ee-2a activity are classified as Ee-2a, whereas those lacking this activity are considered as Ee-2o. The data are not compatible with sex-linked inheritance because males and females of any mating have similar esterase phenotypes and because reciprocal crosses yield only one phenotype.

The Ee-2 esterase phenotypes of F₂ and BC mice are shown in Table 5. Since no significant differences between the sexes was observed, the data on intercross and backcross progeny were pooled for Table 5. The matings are the same as those analyzed in Tables 2 and 3 for the inheritance of esterases Ee-la and Ee-lb. The data obtained from these crosses are compatible with the hypothesis that a single autosomal dominant gene apparently governs the presence of esterase Ee-2a and the recessive allele determines the absence of this esterase form in homozygous animals.

The locus designation Ee-2 is proposed, with allele \(\text{Ee-2}^a\) resulting in the presence of esterase Ee-2a and allele \(\text{Ee-2}^o\) resulting in the absence of enzymatic activity.

### Table 4

**Distribution of the Ee-2 phenotypes among F₁ hybrids**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Male x Female</th>
<th>Ee-2 erythrocytic esterase phenotype of parents</th>
<th>Number of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>A/He x BALB/c</td>
<td>Me x Fa</td>
<td>Ee-2a x Ee-2a</td>
<td>13</td>
</tr>
<tr>
<td>BALB/c x A/He</td>
<td>Me x Fa</td>
<td>Ee-2o x Ee-2a</td>
<td>18</td>
</tr>
<tr>
<td>A/He x C57BL/10</td>
<td>Me x Fa</td>
<td>Ee-2a x Ee-2o</td>
<td>13</td>
</tr>
<tr>
<td>C57BL/10 x A/He</td>
<td>Me x Fa</td>
<td>Ee-2o x Ee-2o</td>
<td>9</td>
</tr>
<tr>
<td>BALB/c x C57BL/10</td>
<td>Me x Fa</td>
<td>Ee-2o x Ee-2o</td>
<td>10</td>
</tr>
<tr>
<td>C57BL/10 x BALB/c</td>
<td>Me x Fa</td>
<td>Ee-2o x Ee-2o</td>
<td>15</td>
</tr>
</tbody>
</table>

### Table 5

**Classification of intercross and backcross progeny for Ee-2 esterase type**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Ee-2 erythrocytic esterase phenotype of parents</th>
<th>Number of offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ee-2a</td>
</tr>
<tr>
<td>ACF₁ x ACF₁*</td>
<td>Ee-2a x Ee-2a</td>
<td>47</td>
</tr>
<tr>
<td>ABF₁ x ABF₁*</td>
<td>Ee-2a x Ee-2a</td>
<td>140</td>
</tr>
<tr>
<td>CBF₁ x CBF₁*</td>
<td>Ee-2o x Ee-2o</td>
<td>0</td>
</tr>
<tr>
<td>ACF₁ x A</td>
<td>Ee-2a x Ee-2a</td>
<td>7</td>
</tr>
<tr>
<td>ACF₁ x C</td>
<td>Ee-2a x Ee-2o</td>
<td>9</td>
</tr>
<tr>
<td>ABF₁ x A</td>
<td>Ee-2a x Ee-2a</td>
<td>51</td>
</tr>
<tr>
<td>ABF₁ x B</td>
<td>Ee-2a x Ee-2o</td>
<td>36</td>
</tr>
<tr>
<td>CBF₁ x C</td>
<td>Ee-2o x Ee-2o</td>
<td>0</td>
</tr>
<tr>
<td>CBF₁ x B</td>
<td>Ee-2o x Ee-2o</td>
<td>0</td>
</tr>
</tbody>
</table>

* The results of matings involving F₁ mice derived from reciprocal crosses were pooled because no significant differences occurred.
activity at the Ee-2a site if present in the homozygous state. According to this theory, animals of type Ee-2a are either Ee-2a/Ee-2a or Ee-2a/Ee-2a. Attempts to correlate staining intensity of Ee-2a and zygosity of mice at the Ee-2 locus were unsuccessful.

**Linkage relationships of Ee-1 and Ee-2.** Table 6 demonstrates the independence of the Ee-1 and Ee-2 esterase types. It is concluded that erythrocytic esterase loci Ee-1 and Ee-2 segregate independently and probably belong to different linkage groups.

Several genes were seen to segregate in the offspring produced by the various crosses of the present study. In an attempt to assign the Ee-1 and Ee-2 loci to specific linkage groups, segregation of five marker genes involving coat color (c, b and a), hemoglobin pattern (Hb) and histocompatibility-2 (H-2) type has been observed in the relevant intercross and backcross offspring. Most of the mice in which the esterase genes were segregating were also classified for these five other inherited traits. The data indicate that the erythrocytic esterase loci are not closely linked to the hemoglobin (Hb, linkage group I), albino (c, I), brown (b, VIII), or histocompatibility-2 (H-2, IX) loci and that Ee-1 is not closely linked to the nonagouti locus (a, V). Thus it appears that Ee-1 and Ee-2 do not belong to linkage groups I, VIII, or IX unless the markers are separated from the erythrocytic esterase loci by more than 50 map units. In addition, Ee-1 does not belong to linkage group V unless it is near or outside of Sd [Danforth’s short tail which is approximately 55 map units from a (Green 1963)] in V.

**DISCUSSION**

**Characterization of esterases:** With the exception of the erythrocytic carbonic anhydrases of some mammalian species (Tashian 1965) the physiological role of red cell esterases is unknown. Therefore, esterases are classified arbitrarily according to their artificial substrate specificities and their sensitivities to various inhibitors and activators.

Acetyleresterases Ee-1a and Ee-1b differ from the A-esterases of primate red cells (Tashian 1965) in being inhibited by DFP. The Ee-1a form probably corre-
sponds to the fastest of the three rapidly migrating esterases reported for C57 Brown Swiss male mice (Templeton 1964, and personal communication).

Esterase Ee-2a is similar to the propionylesterases of primate erythrocytes with respect to order of substrate specificities and eserine sensitivity (Tashian 1965). Unlike the propionylesterases of primate red cells, Ee-2a is not the most electronegative esterase under the present conditions. On the basis of electrophoretic migration, staining intensity with α-naphthyl butyrate, sensitivity to eserine and resistance to DFP, the Ee-2a esterase may correspond to band 1 from hemolysates of Pallid and Swiss Albino mice (Hunter and Strachan 1961).

Esterase Ee-2a is not considered an acetylcholinesterase because the specific anti-acetylcholinesterase agent B.W. 284C51j dibromide was without effect on the activity of this esterase type. If acetylcholinesterase is present at all, it is probably bound to the stroma.

Genetic control of the esterases: The inability to detect an alternate Ee-2 esterase in hemolysates from animals of the genotype Ee-2⁺/Ee-2⁺ may reflect nonexpression of the Ee-2⁺ allele, or that the alternate enzyme is inactive or cannot be detected under the experimental conditions used in the present study. The possibility of regulation of esterase Ee-2a activity by control or suppressor genes cannot be excluded. Esterase Ee-2a is not rendered inactive by an inhibiting factor occurring in Ee-2⁺/Ee-2⁺ hemolysates because mixtures of such Ee-2⁺/Ee-2⁺ lysates and samples exhibiting Ee-2a activity display esterase Ee-2a.

Zymograms from Ee-2⁺/Ee-2⁺ heterozygous mice display an Ee-2a esterase similar in staining intensity to the enzyme of Ee-2⁺/Ee-2⁺ homozygotes. If the staining intensity of the esterase zones roughly approximates enzymatic activity, then the lack of correlation between staining intensity of Ee-2a and zygosity at Ee-2 suggests that there is no gene dosage effect of Ee-2. However, quantitative data on Ee-2a esterase activity from homozygous (Ee-2⁺/Ee-2⁺) and heterozygous (Ee-2⁺/Ee-2⁻) mice are not available. Popp and Popp (1962) have observed differences in total esterase activity associated with the zymogram patterns of inherited serum esterase forms among strains of mice.

Although the possibility exists that the erythrocytic esterase loci Ee-1 and Ee-2 may be identical to one of the mouse esterase loci which determine certain serum (Popp and Popp 1962) and kidney (Ruddle and Roderick 1965) esterases, no genetic evidence is available as yet to establish this relationship. Ruddle and Roderick (1965) report that the eserine sensitive esterase from mouse kidney determined by the Es-3 locus could not be detected in red cells and serum of mice possessing this kidney esterase.

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SUMMARY

Up to 11 zones of nonspecific carboxylic esterase activity were resolved from red cell lysates of the house mouse by the zymogram technique. Certain of these esterase forms were further characterized as acetylenic, acetylpropionyl esterases, and propionyl esterases on the basis of their substrate affinities and differential responses to various activator or inhibitor compounds. Two electrophoretic forms of the Ee-1 acetylenic esterases (Ee-1a and Ee-1b) occur among the 11 inbred strains and three stocks of mice examined. These mice also differed in the presence (Ee-2a) and absence (Ee-2o) of propionyl esterase Ee-2a. Two unlinked autosomal genes, designated Ee-1 and Ee-2, are responsible for the production of the Ee-1 and Ee-2 esterases. Two codominant alleles, Ee-1a and Ee-1b, specify the Ee-1a and Ee-1b esterases, respectively. A single autosomal dominant gene, Ee-2a, governs the presence of esterase Ee-2a and the recessive allele, Ee-2o, determines its absence. Neither erythrocytic esterase locus is closely linked to the hemoglobin (Hb, linkage group I), albino (c, I), brown (b, VIII) or histocompatibility-2 (H-2, IX) genes, nor is Ee-1 closely linked to non-agouti (a, V).

LITERATURE CITED


