IN recent years considerable interest has developed in the genetic control of protein determination in metazoan species. In the house mouse the genetic specification of histocompatibility antigens (SNELL 1963; KLEIN 1963), gammaglobulin (HERZENBERG, MISHELL, and HERZENBERG 1963), and hemoglobins (RUSSELL and GERALD 1958; POPP 1963) have been carefully studied, and a number of distinct mutant forms of these proteins have been described.

The inheritance of another class of proteins—the enzymes—has been studied, and a number of mutant forms have been found. For example, SHAW and BARTO (1963) discovered a lactic dehydrogenase mutant in Peromyscus. Also, several serum esterase mutants have been reported by POPP and POPP (1962), and by PETRAS (1963), in the house mouse. Such mutant proteins can be detected by alterations in electrophoretic mobility, reaction rates, heat sensitivity, and sensitivity to chemical inhibitors.

The work to be described here presents evidence for variant forms of soluble esterases extracted from mouse kidney tissue. It has been possible by means of starch gel electrophoresis to recognize two esterases, A and B, which are native to mouse strain RF/J, and an esterase C, native to C57BL/6J. By means of genetic analysis it has been possible to determine the nature of genetic control for esterases A and B, and preliminary evidence has been obtained for esterase C.

In the majority of the experiments relating to the detection and description of enzyme mutants, sera or erythrocytes have been employed as an enzyme source. In the work described here, the esterases were extracted from adult mouse kidney tissues. Kidney was purposely selected, since this tissue can be cultivated in vitro.

MATERIALS AND METHODS

The animals used in these experiments were inbred strains RF/J and C57BL/6J mice. For the most part, tissues from 6- to 10-week old animals were analyzed, however tissues from much older animals (11 months) showed electrophoretic patterns indistinguishable from those of the younger animals. The treatment of animals and the preparation of the extracts were carried out as uniformly as possible.

Whole kidney and liver extracts were made from individual mice. The organs were cleanly dissected, and care was taken to remove mesenteries and fascia. The organs were placed in normal saline at 4°C for 1 to 5 hours, washed in distilled water, and homogenized with a Porzera-
Elvehjem tissue grinder using an equal volume of distilled water. The homogenate was frozen and thawed three times without permitting the temperature to exceed 4°C. The particulate component was separated from the extracted water soluble fractions in a refrigerated centrifuge at 30,000 g for 30 minutes. The supernatant was collected and subjected to a second similar centrifugation. The extract was stored at -20°C in ampuls until used. Samples were usually analyzed within two months of preparation, with an extension as great as eight months in some instances. Vertical starch gel electrophoresis was performed in an apparatus similar to that described by Smithies (1959). The discontinuous buffer system of Poulak (1957) was employed. The electrode buffer was composed of boric acid (0.29M) and NaOH (0.05M) at pH 8.0. The gel buffer was composed of Tris (0.076M) and citric acid (0.005M) at pH 8.65. The pH of these solutions was checked before each run. Electrophoresis was executed at 4°C in most instances, with electric fans to dissipate heat. Running time was 7 to 8 hours at a constant 460 volts. Hydrolyzed potato starch prepared by Conaught Laboratories was employed at a concentration (14 percent) somewhat greater than recommended by the supplier. For some purposes an increase to 16 percent was employed. It was found that higher starch concentrations permitted a better separation of esterase A (see below), while lower concentrations favored resolution of esterase B.

The esterases were stained by coupling the reaction products of naphthyl substrates with a diazonium salt. The naphthyl substrates were alpha-naphthyl acetate (Sigma; 75 mg/150 ml reaction mixture) and alpha-naphthyl butyrate (Sigma; 0.02 ml/150 ml reaction mixture). The naphthyl substrates were dissolved in 1 ml of acetone before addition. Fast blue BB (Dajac; 750 mg/150 ml reaction mixture) was used as the diazonium salt. Gels were incubated 20 min before staining in phosphate buffer (0.05M, pH 7.4) to which had been added CaCl₂ (10⁻³M Ca++. The buffers employed for incubation and staining were identical. Staining reactions were run 30 min at 25°C for alpha-naphthyl acetate, and 90 min for alpha-naphthyl butyrate. Eserine sulfate (K and K; 10⁻³M) was incorporated in both the incubation and staining mixtures whenever employed.

Following the staining, gel slices were fixed in a mixture of methanol, glacial acetic acid, and water (50:20:50). Electrophoresis patterns were photographed with both Land polaroid film and Kodak contrast process panchromatic film.

RESULTS

Differences in banding patterns in three sets of esterases in Zone IV were detected between inbred mouse strains C57BL/6J and RF/J. We have termed these esterases A, B, and C (Figure 1). Esterase A, found only in RF/J mice, migrates more rapidly than the other two esterases (Figures 2 and 3). Esterase B, also found only in RF/J animals, migrates in the cathodal region of Zone IV (Figures 2 and 3). Esterase C, found only in C57BL/6J mice, has the lowest electrophoretic mobility in Zone IV (Figures 2 and 3). These enzymes can be distinguished by their different pharmacological properties as well as by their relative mobilities (Ruddle, unpublished). Esterase A was inhibited by eserine sulfate at 10⁻³M level on the starch gel and also has many of the properties of a nonspecific pseudocholinesterase (Ruddle, unpublished). There was a tendency for stronger bands of esterase A in males than in females (Figures 2 and 3). Esterase B was not inhibited by eserine and was perhaps slightly activated by it. Esterase B has the properties of an aliesterase (Ruddle, unpublished). Enzyme C was also classified as an aliesterase but differs from other aliesterases by its great lability. This enzyme was inactivated by storage for two months at -20°C. It was inactivated in 30 minutes at 37°C (Ruddle, unpublished).

The genetic evidence is based on differences in banding patterns observed in
Figure 1.—Schematic representation of mouse kidney esterases obtained by means of vertical starch gel electrophoresis. Detailed banding pattern is shown only for Zone IV esterases. Esterases designated by letters from the beginning of the alphabet are found either in RF/J or C57BL/6J extracts. Esterases designated X₁ and X₂ are common to both strains of mice.

strains RF/J, C57BL/6J, their F₁, first (BC₁) and second (BC₂) backcrosses to C57BL/6J, and first backcross to RF/J. More data are available on the inheritance of enzymes A and B than on enzyme C. Since the second backcross to RF/J animals was not produced, the evidence for the control of enzyme C by a single gene is considered as preliminary.

Zymograms of extracts of F₁ kidney stored for only a few days are shown in Figures 2 and 3. These samples show the presence of all three enzymes. The fast band of the C esterase set and that of band B overlap, or very nearly overlap (the B esterase may migrate somewhat faster), and thus obscure one another. The presence of band B can be seen in the absence of C after aging of the extract (Figure 5).

In the offspring of the backcrosses of F₁ hybrids to RF/J mice all extracts were positive for esterases A and B (Table 1; Figure 4). There was also an indication of a difference in intensity between A bands in these animals. The numbers of A bands of strong and weak staining intensity in this family were six and five respectively.

Offspring of the backcross of F₁ hybrids to C57BL/6J showed a segregation of esterases A and B (Table 1). Since these extracts were studied several months after their preparation, it was not possible to score the activity of C esterase. For enzyme A the results closely approximate a 1:1 ratio of plus to minus animals. For enzyme B there is a statistically significant departure from a 1:1 ratio, but this deviation is presumed to be due to chance and therefore probably not bio-
MOUSE KIDNEY ESTERASES

TABLE 1

The number of animals which have (+) or do not have (−) esterases A and B in two strains of mice and various crosses between them

<table>
<thead>
<tr>
<th>Strain or cross</th>
<th>Enzyme</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF/J</td>
<td></td>
<td>68:0</td>
<td>68:0</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td></td>
<td>0:90</td>
<td>0:90</td>
</tr>
<tr>
<td>F₁ (of above)</td>
<td></td>
<td>10:0</td>
<td>10:0</td>
</tr>
<tr>
<td>BC₁ to C57BL/6J</td>
<td></td>
<td>15:16</td>
<td>22:9*</td>
</tr>
<tr>
<td>BC₁ to RF/J</td>
<td></td>
<td>22:0</td>
<td>11:0</td>
</tr>
<tr>
<td>BC₂ to C57BL/6J</td>
<td>(a) where BC₁ parent was +</td>
<td>40:47</td>
<td>57:66</td>
</tr>
<tr>
<td></td>
<td>(b) where BC₁ parent was −</td>
<td>0:74</td>
<td>0:23</td>
</tr>
</tbody>
</table>

* χ² = 5.43, P = 0.02, a statistically significant deviation from a 1:1 ratio.

logically significant. The deviation is attributable to the sample of BC₁ animals which were used for parents for the BC₂ (Table 2). Further information from the BC₁ animals do not corroborate the deviation. Segregation of B esterase in BC₁ kidney extracts is shown in Figure 5 and in BC₂ extracts in Figure 6.

The second backcross (BC₂) to C57BL/6J permitted a further test of the

**Figure 2.**—F₁ hybrid esterase patterns, Zone IV. Extracts 1–3: RF/J; extracts 4–7: F₁ hybrids of RF/J × C57BL/6J; extracts 8–10: C57BL/6J. Extracts 4–10 were run within 4 days of their preparation. Note A and B bands in RF/J controls and F₁ hybrids. The A bands are typically more pronounced in male F₁ extracts (samples 4 and 5). Note C bands in C57BL/6J extracts (samples 8–10).

**Figure 3.**—F₁ hybrid esterase patterns, Zone IV. Extracts 1 and 2: RF/J; extracts 3–8: F₁ hybrids of RF/J × C57BL/6J; extract 9: C57BL/6J. Extracts 3–8 were run within 4 days of their preparation. Extract 9 was run two months after its preparation. Note A and B bands in RF/J extracts, C and A bands in F₁ hybrids, and the absence of A, B, and C bands in the C57BL/6J extract. Loss of C bands is characteristic of extracts stored for more than 2 months at −20°C. Note enhanced activity of A enzyme in male extracts 6–8.

**Figure 4.**—Segregation of C esterase bands, Zone IV. Extract 1: F₁ hybrids of RF/J × C57BL/6J; extracts 2–9: offspring of F₁ hybrids × RF/J (BC₁); extract 10: RF/J. Extract 1 was run 2 months after its preparation. Extracts 2–9 were run within 4 days of their preparation. Note the presence of A and B bands in all patterns and the segregation of the C band. The C band is scored as positive in samples 3, 4, 5, 7, and 8.

**Figure 5.**—Segregation of B esterase bands, Zone IV. Extract 2: C57BL/6J; extracts 3–9: offspring of F₁ hybrid × C57BL/6J (BC₁); extract 10: RF/J. The BC₁ extracts were run 2 months after their preparation. The gel was treated with eserine sulfate (10⁻³M) in order to inhibit the A esterase bands. The B band is scored as positive in samples 1, 3, 5, 7, 8, and 10.

**Figure 6.**—Segregation of A esterase bands, Zones IV and V. Extract 2: C57BL/6J; extract 9: RF/J; extracts 1, 3–8, and 10: offspring of BC₁ × C57BL/6J (BC₂). Zone V was included in order to help visualize the A esterase bands. Note the presence of A esterase bands in RF/J, absence in C57BL/6J, and segregation in the BC₂ samples. Fluctuation in the cathodal end of Zone IV is due to segregation of the B enzyme. This band does not show clearly in gels of high starch concentration. The A esterases are scored as positive in samples 5, 7, and 9. The B esterases are scored as positive in samples 4, 5, 9, and 10.
hypothesis of a single-gene basis for the inheritance of the enzyme patterns. Six BC₁ males and six BC₁ females (F₁ × C57BL/6J) were mated to C57BL/6J animals. The BC₁ parents were coded, and their relationships to their offspring were not revealed until the offspring had been analyzed. If the presence of A and B esterases were determined by dominant RF/J genes, then those enzymes must be present in the BC₁ parent if they are to be found in any of the BC₂ offspring of that parent. Conversely, if an enzyme were found in the BC₂ offspring, then it must also be present in the BC₁ parent. For esterase A the enzyme patterns of the BC₁ parents were predicted correctly for all 12 litters (Table 2).

A further prediction, if an enzyme were determined by a single dominant gene, is that in those BC₂ litters which do have at least one plus animal, the plus and minus animals should appear in approximately equal numbers. One would have to test sufficient offspring from all matings so that the probability would be negligible of producing by chance all minus animals when the BC₁ parent was plus. A minimum of eight animals in the BC₂ was tested for each mated pair; the probability by chance of producing eight minus animals where one would expect equal numbers of plus and minus animals is (1/2)^8 which is less than .004. The results are in close agreement with this prediction for both enzymes (Table 2).

To determine whether the genes for enzymes A and B are linked, we used the
data from the BC\textsubscript{1} animals as well as all the BC\textsubscript{2} animals which were assayed for both enzymes and whose BC\textsubscript{1} parent was plus for both enzymes. In these animals if no linkage exists, one would expect equal proportions of parental classes A+B\(+\), A−B− and recombination classes A+B− and A−B+. The numbers observed for these classes were respectively 23, 29, 23 and 28 ($\chi^2 = 1.48; P \approx 0.70$). The genes therefore behave as unlinked genes. In crosses in these experiments where the sire was plus for enzyme A and the dam was minus, male progeny were found which were plus for enzyme A. The same results were found in analogous crosses involving enzyme B. There is therefore no evidence for sex linkage of either gene.

Only incomplete data exist at present for the genetic control of the C esterases, because the lability of these enzymes was not clearly recognized until the present study was well advanced. However, it is clear that these esterases are found in C57BL/6J but not in RF/J extracts. Support for the hypothesis of a single gene control for C esterases comes from a 6+:5− segregation ratio of these enzymes in BC\textsubscript{1} individuals resulting from F\textsubscript{1} × RF/J crosses. This segregation pattern is lost when the same extracts are re-run after several months storage at −20°C, because of the inactivation of the C esterase. A new series of experiments will be performed to ascertain the precise genetic basis of C-esterase expression.

While the distribution of the various esterases described above in kidney cell types is not known, evidence does exist that enzyme A is related to some function of the kidney. Examination of RF/J erythrocyte hemolysate and serum did not reveal enzyme A. Trypsin digestion of adult RF/J kidney was carried out to remove parenchyma. The free tubules and glomeruli were washed in physiologic buffer saline and homogenized. Such extracts showed undiminished esterase A activity.

**DISCUSSION**

The techniques employed in the work of Markert and Hunter (1958) and in this experiment are sufficiently similar to allow comparison. The major banding sequence in Markert and Hunter's work probably corresponds quite closely to that reported in these investigations. The main difference is the greater resolution of the method reported here which allows the detection of three to four times the number of distinct bands. Zone I esterases reported here were probably not seen by Markert and Hunter. Zone II esterases and Zone III esterases reported here probably correspond to Markert and Hunter's a, b, c and d, e, f bands respectively. There is strong reason to believe that Zone IV corresponds to g, h, and i, since the g band was inhibited by eserine at a concentration of $10^{-4}$ according to these workers. The eserine inhibited esterase was always found in Zone IV in our studies. Zone V probably is the same as band j.

Markert and Hunter describe an eserine sensitive band in a position which agrees with our Zone IV. In C57BL/6J there is no appreciable inhibition of a distinct esterase in this zone. However, survey studies to be reported elsewhere do indicate an eserine sensitive band in the cathodal end of Zone IV in mouse strains LP/J, A/HeJ, CBA/J, DBA/J, and 129/J. A similar though much weaker
band may also exist in kidney extracts of strains SJL/J and BALB/cJ. These bands agree more closely with the eserine sensitive band reported by Markert and Hunter than with the RF/J esterase A. Esterase A is located in the anodal portion of Zone IV, and in gels of high starch concentration may migrate in the space between Zones IV and V (Figure 6). A genetic analysis of the relationship of the eserine sensitive bands of the above strains to esterase A in the RF/J strain is in progress.

A literature is now accumulating on the genetic control of the esterases in a number of organisms. However, it is as yet too early to detect the emergence of any clear-cut patterns. Roderick (1960) in a study of cholinesterase of the rat brain selected successfully for high and low enzyme activity in two genetically independent heterogeneous populations of rats. This study suggested a polygenic control of direct and/or indirect factors which may influence cholinesterase activity and/or synthesis. Since in this experiment he measured average enzyme activity of tissue extracts as determined by titrimetry, the various possibilities cannot be distinguished. It will be important in further studies of this sort to perform qualitative studies on the enzyme(s) during the course of selection by means of starch gel electrophoresis or some comparable method. By this means it should be possible to detect increases of activity in all the fractionated enzymes or to discern activity differentials between the individual enzymes.

Several investigations have shown, in agreement with this paper, that distinct esterases as revealed by starch gel electrophoresis may have different phenotypic forms or may be lacking completely in certain individuals. These differences may be controlled epigenetically as has been shown by Markert and Hunter (1958) and Allen (1960), or genetically. Investigators have described a number of esterase polymorphisms under simple genetic control at a single genetic locus. Such instances have been reported by Augustinsson and Olsson (1959) for pig arylesterases, Allen (1960) for Tetrahymena, Shaw, Syner, and Tashian (1962) for erythrocyte esterases in man, Wright (1963) and Wright and McIntyre (1963) for Drosophila esterases, Popp and Popp (1962) for esterases in mouse serum, and Petras (1963) for esterases in mouse serum. In all of these studies, there is no evidence that the enzymes involved are composed of subunits whose coding sequence is under the control of different alleles. If isozymes are defined primarily on the basis of subunit interaction, then these above listed enzymes should not in our opinion be termed isozymes at present. The work of Schwartz (1960) on maize presents the only instance in a higher organism for hybrid forms of esterases.

Popp and Popp (1962) noted differences in the esterase banding pattern of C57BL serum in comparison to sera of strains 101, BALB/c, and SEC. They reported that C57BL possessed a single band termed "I" which had no counterpart in the other strains. The other strains possessed two slower migrating bands which were not shared with C57BL. These bands were named "II". These three bands in Figure 9 of Popp and Popp probably correspond to Zone IV reported in the present study. The finding that their "I" band of C57BL migrates in advance of the bands "II" does not match with any of our observations made on
the extracts of mouse kidney. In no case were C57BL bands seen to migrate in advance of the bands in other strains, including especially BALB/cJ. POPP and POPP did not mention the effect of eserine on either bands I or II and so no comparison between studies in this respect can be made. A preliminary study on the sera of RF/J mice failed to detect the presence of any enzyme which might be esterase A.

The data presented here have indicated clearly the presence of two independently segregating loci for the A and B esterases. In this respect our results differ from the conclusion of POPP and POPP that their bands “I” and “II” are determined by two alleles at the same locus. Thus there are similarities and differences between their work and our own. It should be emphasized that serum was analyzed in their study and kidney extract in ours. Also, technical procedures employed in the two studies were quite different.

PETRAS (1963) has described another instance of genetic control of a serum esterase in feral Mus musculus. He showed that mutant mice may lack band I (PETRAS’s terminology), and that the expression of this band is determined by a pair of autosomal codominant alleles at a single locus. The esterase reported by PETRAS may be identical to one of those which contributes to Zone V reported here. In our studies no variation was seen with regard to Zone V.

Without evidence that the kidney esterases in this study correspond with the serum esterases of POPP and POPP (1962) and PETRAS (1963), we tentatively designate the locus for enzyme A with the symbol Es-3 and the locus for enzyme B with the symbol Es-4. The alleles at these loci are represented by letter superscripts as follows: \( Es-3^a \), the allele producing no expression of enzyme A; \( Es-3^b \), the allele producing the expression of enzyme A; \( Es-4^a \), the allele producing no expression of enzyme B; \( Es-4^b \), the allele producing the expression of enzyme B. This genetic nomenclature follows that of POPP and POPP (1962) and PETRAS (1963) and is in accord with that recommended by The Committee on Standardized Genetic Nomenclature for Mice (1963).

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**SUMMARY**

Three kidney esterases, two of which are native to RF/J mice (esterases A and B) and one which is native to C57BL/6J (C esterase) have been distinguished by vertical starch gel electrophoresis. Esterase A is further characterized by its inhibition by eserine sulfate \( (10^{-3} \text{M}) \), and esterase C has been shown to be highly susceptible to temperature inactivation. The inheritance of these traits in the descendants of RF/J and C57BL/6J parents indicates that enzymes A and B are determined by dominant genes at separate loci. There was no indication of linkage between the two loci or of hybridization between the different enzymes. The
loci which control the expression of enzymes A and B have been given the symbols $Es$-3 and $Es$-4, respectively. Preliminary evidence suggests that enzyme C is also determined by a single locus.

LITERATURE CITED


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