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Electrophoretic variants in erythrocytes of the house mouse, Mus musculus.

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ELECTROPHORETIC VARIANTS IN ERYTHROCYTES OF THE HOUSE MOUSE, MUS MUSCULUS

BY

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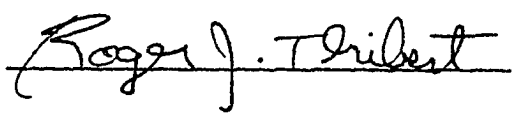

ABSTRACT

Using starch-gel electrophoresis and appropriate staining, four variants have been found in erythrocytes from the house mouse, Mus musculus.

An allele, designated Es-3^{d}, controlling the presence of a fast-migrating esterase (designated Es-3d) has been found at the Es-3 locus. Tests of substrate specificity, heat sensitivity, and of eserine sulphate and iodoacetamide sensitivity, showed Es-3d and two other esterases (Es-3b and Es-3c) controlled by the Es-3 locus to react identically. The frequency of Es-3^{d} is low (0.02 - 0.05) in natural populations of the house mouse. It is suggested that amino acid substitutions at one site of the protein could account for the electrophoretic patterns of Es-3b, Es-3c, and Es-3d.

By carrying out starch-gel electrophoresis in Tris-maleate-NaOH buffer, pH 7.0, an improved resolution of house mouse hemoglobin has been obtained. The binding of maleate to a free -SH group on the diffuse hemoglobin is postulated to be the cause for the charge difference between diffuse and single hemoglobins. Several hypothesis are presented as explanations for the electrophoretic patterns observed when diffuse hemoglobin is mixed with various sulfhydryl reagents.

Two lactate dehydrogenase (LDH) variants have also been found in house mouse erythrocytes. The first involves the presence and absence of red blood cell LDH isozyme four. The presence of LDH IV is in high frequency (0.80) in the wild mice studied. The variant seems to be under the control of locus Ldr-1 reported by Shows and Ruddle (1968), which itself appears to be the same as the gene reported by Riles (1965). The second LDH variant involved differences between C3H and C57BL/10 LDH V after a three week storage of hemolysates. The electrophoretic patterns of hemoglobin and LDH V before and after treatment indicate that the variant could be a reflection of the concentration of a storage product, such as oxidized glutathione.

The proteins which were studied showed differences in charge which are believed due to (a) amino acid substitutions at one site of the protein, (b) substitution of one amino acid by another which itself does not possess a charge at most physiological pH's, but is quite reactive with sulphur containing compounds and other reagents, (c) the gene-regulated absence of a polypeptide subunit, or (d) interaction between the protein and a charged storage product.

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CHAPTER 1

INTRODUCTION

The process of separating charged molecules by passing them through an electric field is called electrophoresis. Smithies (1955) modified the earlier electrophoretic techniques by using starch-gel as the supporting medium. This permits a relatively rapid and distinct separation of differently charged molecules, such as proteins. Since the net charge of a protein varies with the hydrogen ion concentration of its environment, a buffer is used to maintain a constant charge on the various protein molecules. This constant charge results in a reproducible electrophoretic separation of various proteins. A second factor which effects protein separation in starch-gel is molecular size. The more concentrated gel decreases the migration rate of large molecules (see Smithies, 1955).

At this point it may be useful to review the basis for the differences in charge found among proteins. Proteins are macromolecules composed of a large variety of amino acids arranged linearly. Amino acids are those organic compounds which possess at least one carboxyl ($-COO^-$) and one amino group ($-\overset{+}{N}H_3$) per molecule. Since amino acids bind to form proteins by forming peptide ($-\overset{O}{\overset{\|}{C}}-\overset{H}{\overset{|}{N}}-$) bonds, one carboxyl group (negatively charged) and one amino group (positively charged) are eliminated from the polypeptide per amino acid

bound. Also, the charge of the carboxyl group at one end of the protein molecule essentially neutralizes the charge of the amino group found at the other end. Thus, if amino acids had only one carboxyl and one amino group, the protein (amino acid polymer) would possess a neutral charge at most physiological pH's. However, this is not always the case. There are some amino acids which have either additional carboxyl groups (acidic amino acids) and/or additional amino groups (basic amino acids). It is the presence of amino acids containing additional carboxyl or amino groups which gives a protein its net charge at a particular pH.

During the course of the present study, the following four causes of charge differences between proteins are presented:

1) Specific differences in charges due to amino acid substitution.

The presence of a fast migrating erythrocytic esterase has been found in some wild mice. The esterase has been found to be controlled by an undescribed allele at the Es-3 locus (Popp, 1966). The three detectable esterases controlled by alleles at the Es-3 locus were found to behave identically to tests of substrate specificity and of heat, iodacetamide, and eserine sulphate sensitivity. However, they migrate at different rates during electrophoresis through a buffered starch-gel. The ability to separate these esterases electrophoretically appears to be due to an

inherent difference in their amino acid composition.

2) Substitution of one amino acid by another which itself does not possess a charge at most physiological pH's, but is quite reactive with sulphur containing compounds and other reagents.

This fairly specific reactivity is postulated to be the basis for an electrophoretic technique which improves the resolution of mouse hemoglobins. The usual technique for typing mouse hemoglobin (Popp, et al., 1960) gave three electrophoretic patterns, two of which (homozygous diffuse and heterozygous diffuse) were at times scarcely distinguishable. With a pH 7.0 Tris-maleate - NaOH buffer, the three hemoglobin phenotypes were found to be easily identified. Studies using various sulfhydryl reagents indicate that the maleate component of this buffer is reacting with a free -SH group on the diffuse hemoglobin of C3H mice, thereby increasing the net charge of the hemoglobin. Several hypotheses are presented which could explain the electrophoretic patterns obtained when diffuse hemoglobin is mixed with other sulfhydryl reagents.

3) The gene-regulated absence of a differently charged polypeptide subunit.

After electrophoresis and staining of some mouse tissues, five isozymes or zones of lactate dehydrogenase (LDH) activity appeared (Markert and Ursprung, 1962). Markert (1968) has suggested that the LDH isozymes

represent the five possible tetramers which would be formed by the random association of two differently charged polypeptide subunits. The first of two electrophoretic variants of erythrocytic murine lactate dehydrogenase involves the presence or absence of one of the LDH isozymes. The absence of this band appears to be due to a gene regulating the synthesis or release of one of the polypeptide subunits.

4) Interaction between a protein and a charged storage product.

The second of the two LDH variants involves a difference in the migration rate of the most cathodal LDH isozyme in two inbred strains of mice. The latter appears to reflect differences in availability of a charged storage product found in the lysates of the two inbred strains.

Also, results of matings established to test for the modes of inheritance of the variants and the frequencies of their alleles found in several populations of wild mice are presented.

CHAPTER II

STUDIES OF THE Es-3 - CONTROLLED ESTERASES IN ERYTHROCYTES

Hunter and Markert (1957) were the first to demonstrate that the enzymatic composition of tissues could be analyzed by combining histochemical techniques with zone electrophoresis. In 1959 Markert and Hunter reported on the distribution of esterases in thirty-two tissues of the house mouse, including the blood. Subsequently, a number of inherited esterase variants have been found in house mouse (Mus musculus) serum (Popp and Popp, 1962; Petras, 1963; Petras and Biddle, 1967), red blood cells of the house mouse (Pelzer, 1965; Popp, 1966) and kidney cells (Ruddle and Roderick, 1965; Petras and Sinclair, 1969).

One of the esterase variants found in mouse kidney homogenates was described by Ruddle and Roderick (1965) who proposed that the locus controlling this variant be designated as Es-3. Almost simultaneously, Pelzer (1965) reported an erythrocytic esterase variant in inbred strains of mice which he described as being controlled by locus Ee-2. Popp (1966) showed that loci Es-3 and Ee-2 are identical and suggested that this esterase-controlling locus be referred to as Es-3, since this designation had chronological priority.

Three alleles have been reported at the Es-3 locus

(Ruddle and Roderick, 1965; Popp, 1966). The absence in C57BL/10 mice of any detectable gene product (Es-3a) was proposed to be the result of the Es-3^a allele in the homozygous condition. Recently, Ruddle and Roderick (1968) have reported results which "provide yet another instance of detection of the product of a so-called 'silent allele", (i.e., Es-3a). Es-3^c is the allele present in C3H mice, in which it is responsible for the production of an esterase, detectable in both kidney and red blood cells as a distinct band (referred to as Es-3c) after starch-gel electrophoresis and appropriate staining. The presence of a faster migrating esterase band (Es-3b) found in most wild mice and some inbred strains (Popp, 1966) is controlled by the Es-3^b allele.

During the typing of wild house mice (Mus musculus) trapped on farms south of Windsor, Ontario, some animals had both an Es-3b band and also a slightly faster anodal-migrating erythrocytic esterase. Matings between three wild females and C3H males, and subsequently their offspring, were established to test for the mode of inheritance of this fast-migrating esterase. Results of these matings, together with the results of tests involving substrate specificity, heat sensitivity, and eserine sulphate and iodoacetamide sensitivity of this esterase, suggest that this faster enzyme is controlled by yet another allele of the Es-3 locus.

Materials and Methods

The techniques for bleeding the mice and preparing the erythrocytic lysates have been described by Biddle and Petras (1967). Electrophoresis was routinely carried out using a 16% (w/v) starch gel prepared from hydrolyzed potato starch (Connaught Laboratories, Toronto, Ontario), and the discontinuous buffer system (Tris-citrate, borate-NaOH; pH 7.5) described by Kristjannson (1963). The gel was prepared as described by Biddle and Petras (1967) and the samples were placed in slots. Electrophoresis was carried out using a voltage gradient of 10 V/cm, at a temperature of 2-4°C. A small fan circulated air over the gel to increase heat dissipation. After the borate band had migrated 10 cm. (about 3½-4 hours), the gel was removed from its tray and sliced horizontally into two layers.

The staining procedure was a modification of that described by Pelzer (1965). Ten ml. of 0.1 M solutions of α - naphthol (Sigma), α - naphthyl acetate (Eastman Organic Chemicals), α - naphthyl proprionate (Sigma), α - naphthyl butyrate (Sigma), α - naphthyl valerate (Sigma), α - naphthyl caprylate (Sigma), α - naphthyl myristate (Sigma), β - naphthyl acetate (Sigma), naphthol AS-D acetate (Sigma), α - naphthyl stearate (Sigma) were made up in acetone (Fisher). Also, a 0.1 M solution of the sodium salt of α - naphthyl acid phosphate (Sigma) was prepared in distilled water. These solutions were stored at 2°C and used in 5 ml.-quantities with 20 ml. of 0.4 M pH 7.1 Tris-HCl

buffer (Pelzer, 1965) and 175 ml. distilled water in which 200 mg. of Fast Blue BB salt (Sigma) had been dissolved. All staining was done at room temperature (20-22°C).

Esterase sensitivity to heat (58.5°C for twenty minutes) to eserine sulphate (10^{-3} M in stain buffer for thirty-six minutes at room temperature) and to 3.3×10^{-2} M iodoacetamide (in stain buffer for thirty-six minutes at room temperature), was determined. Appropriate controls were run.

Results

The electrophoretic pattern of the fast esterase (F.E.), in relation to the position of Es-3b and Es-3c, is demonstrated in Figure 1. The fast esterase appears as a distinct band migrating towards the anode. Es-3c appears as a distinct band located cathodally to the fast esterase. Es-3b has migrated to a position midway between the fast esterase and Es-3c.

The results of matings set up to test for the mode of inheritance are seen in Table 1. They are consistent with a one-locus, three-allele mode of inheritance.

The time at which the bands of stain appeared on the gel was used as a crude indication of the specificity of the esterase for a particular substrate. The substrate which appeared to be broken down the fastest was α- naphthyl proprionate. The following substrates are given in order of appearance of the bands: α - naphthyl acetate = α - naphthyl butyrate, naphthol AS-D - acetate, β - naphthyl

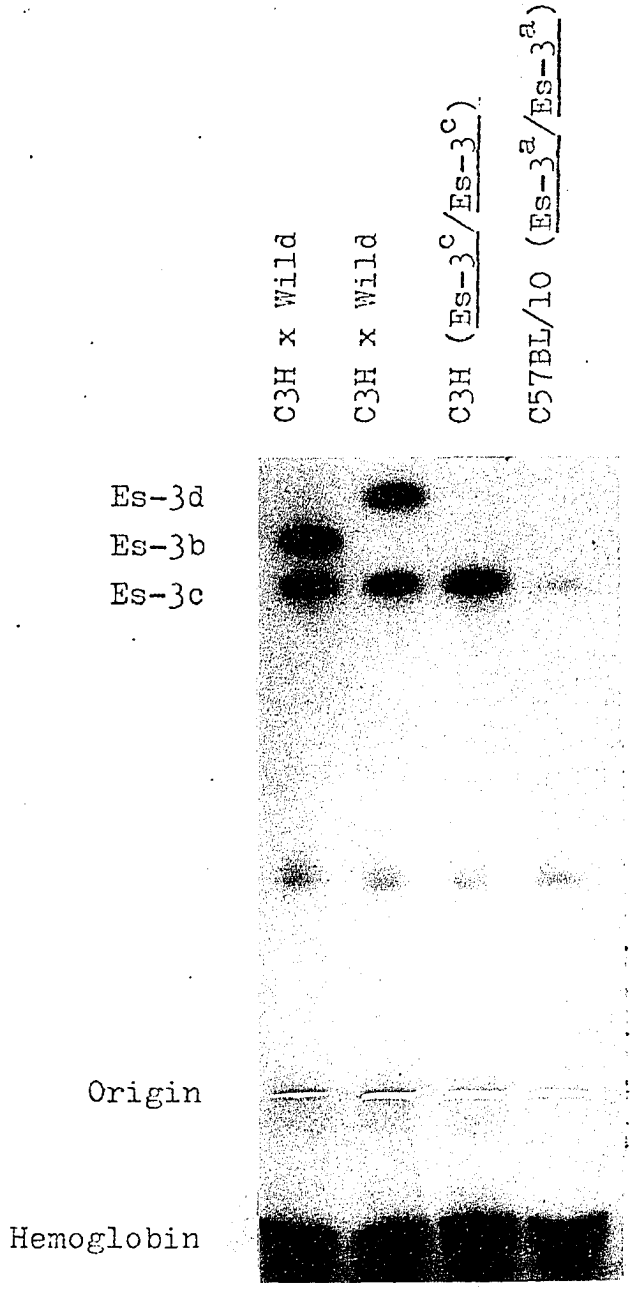

FIGURE 1: Patterns of the Es-3 - controlled esterases after electrophoresis (Kristjannson, 1963) and staining (Pelzer, 1965).

TABLE 1

Breeding Data from Matings Set Up to Test
for the Mode of Inheritance of the Fast Esterase (F.E.) Variant

Matings	Phenotypes				X^{2*}	P
	Es-3b + Es-3c	F.E. + Es-3c	F.E. + Es-3b	Es-3c		
1) ♂ C3H (Es-3c) x ♀ Wild (Es-3b + F.E.)	3	3	-	-		
2) ♂ F_1 (Es-3b + Es-3c) x ♀ F_1 (Es-3c + F.E.)	11	6	5	4	3.60	>0.30
3) ♂ F_1 (Es-3b + Es-3c) x ♀ C3H (Es-3c)	16	-	-	9	1.96	>0.10
4) ♂ C3H (Es-3c) x ♀ F_1 (Es-3c + F.E.)	-	15	-	7	2.90	>0.09

* Based on a lone-locus, three-allele mode of inheritance.

acetate, and α- naphthyl valerate. Several substrates of higher molecular weight including α - naphthyl caprylate (M.W. = 270), α- naphthyl myristate (M.W. = 354), and α - naphthyl stearate (M.W. = 411), did not appear to be broken down by the Es-3 esterases even after twenty-eight hours of incubation. Since α - naphthyl stearate would not dissolve in water and would only form a suspension in acetone, the conclusions about it may not be valid. The bands observed when the sodium salt of α - naphthyl acid phosphate was used as substrate did not correspond in either position or intensity to those found with the other substrates. They appeared as faint zones of activity with each zone being composed of two closely associated bands which migrated at about the same rate as Es-3c.

Incubation of the gel in buffer at 58.5°C for twenty minutes as described by Pelzer (1965) did not affect the activity of the esterases with α - naphthyl acetate as the substrate.

Preincubating a gel slice for thirty-six minutes in 3.3 x 10^{-2} M iodoacetamide did not affect the final intensity of the bands. However, treatment with 10^{-3} M eserine sulphate (physostigmine) for thirty-six minutes decreased the rate of staining and the intensity of the zones over those observed in the control.

Discussion

From the results of mating two (Table 1), several

conclusions can be drawn. When Es-3b and Es-3c were tested for being controlled by two different loci, a X^2 of 4.59, with three degrees of freedom ($P > 0.20$) was obtained (this result was probably due to a small sample size). When Es-3b and Es-3c were studied for allelism, a X^2 of 4.14 with two degrees of freedom ($P > 0.10$) was obtained. When Es-3c and the fast esterase were tested for being controlled by two different loci, a X^2 of 8.55 with three degrees of freedom ($P < 0.05$) was obtained. When Es-3c and the fast esterase were studied for allelism, a X^2 of 0.68 with two degrees of freedom ($P > 0.70$) was obtained. When Es-3c, Es-3b, and the fast esterase were studied for allelism, a X^2 of 3.58 with three degrees of freedom ($P > 0.30$) was obtained. This indicates that the three esterases, Es-3b, Es-3c, and the fast esterase are controlled by three alleles at the Es-3 locus. The possibility that two closely-linked loci, one which controls the fast esterase and the other, the Es-3 locus, are responsible for the results cannot be eliminated at this time. Thus, the allele controlling the presence of the fast esterase is tentatively designated $Es\text{-}3^d$, with the fast esterase controlled by $Es\text{-}3^d$ being designated Es-3d.

In order to compare the esterases controlled by the Es-3 locus, several parameters were studied. Hemolysates containing the products of the four known alleles [$Es\text{-}3^a$ (found in the homozygous condition in C57BL/10 mice), $Es\text{-}3^b$ (found in high frequency in wild mice), $Es\text{-}3^c$ (found in the homozygous condition in C3H mice), and $Es\text{-}3^d$ (found in low

frequency in wild mice)], were run side by side on the same gel. All esterases examined were inactivated to a considerable degree by exposure to 10^{-3} M eserine sulphate. This is consistent with the results of Pelzer (1965), Popp (1966), and Ruddle and Roderick (1965). Exposure to 3.3×10^{-2} M iodoacetamide had no affect on the activity of the esterases. Heat inactivation did not occur when the gel containing the esterases was exposed to 58.5°C for twenty minutes. In the procedure used here, the starch-gel and/or the buffer may be protecting the esterases from heat inactivation.

The "active" esterases, (Es-3b, Es-3c, Es-3d), exhibited an identical specificity for naphthyl substrates. A substrate with the side chain in the α position appears to be broken down faster than a substrate with the side chain in the β position (e.g. α - naphthyl acetate and β - naphthyl acetate). Second, the rate of reaction seems to be correlated slightly with the number of carbon atoms in the side chain. The three carbon proprionyl substrate is broken down the fastest [this is consistent with Popp's (1966) results], with the two and four carbon (α - naphthyl acetate and α - naphthyl butyrate, respectively) being broken down equally slower, and the five carbon substrate (α - naphthyl valerate) giving the slowest reaction recognizable. The higher molecular weight substrates referred to previously gave no visible reaction product. Inability of these substrates to dissolve in an aqueous medium may account for these results.

To summarize, no differences in activity of the Es-3b,

Es-3c, Es-3d esterases were observed after the various treatments discussed above.

The Es-3 locus has been found to be polymorphic in populations of feral mice studied. Table 2 summarizes the frequencies of the alleles at the Es-3 locus over two years of trapping.

Ruddle and Roderick (1968) reported that under their conditions $Es\text{-}3^a$ (homozygous in C57BL/10 mice) is not "silent". The evidence they present is the presence of a kidney esterase band in C57BL/6J which migrates to the same position as other bands from kidney homogenates of mice from their DX-ML heterogenous population. No mating results are presented to establish that the esterase is controlled by an allele at the Es-3 locus. In fact, since they are using a new buffer system (pH 8.6; Tris,-EDTA,-borate) which is quite different from the discontinuous Tris-citrate, borate-NaOH buffer (Poulik, 1957) which they used previously, correlation and linkage data should be presented which proves that the esterases they have found are controlled by the Es-3 locus.

Summary

An esterase, found in low frequency in wild mice, has been shown to be controlled by a new allele, designated $Es\text{-}3^d$ at the Es-3 locus.

Attempts to characterize this new esterase using substrate specificity, temperature sensitivity, and reaction to

TABLE 2

Summary of the Frequencies of Alleles at the Es-3 Locus Found in Wild Mice

Year	Number of Mice	Frequency of Allele			
		Es-3^a	Es-3^b	Es-3^c	Es-3^d
1966	234	*	0.93	0.05	0.02
1968	112	*	0.93	0.01	0.05

* The Es-3^a allele is assumed to be absent in the wild populations studied (see Petras, et al., 1969).

eserine sulphate, and iodoacetamide, have shown it to behave like the other esterases controlled by this locus.

CHAPTER III

A DESCRIPTION AND EXPLANATION OF AN IMPROVED ELECTROPHORETIC RESOLUTION OF MOUSE HEMOGLOBINS

Examination of mouse hemoglobins by paper electrophoresis (Gluecksohn-Waelsch, et al., 1957), and subsequently starch-gel electrophoresis (Popp and St. Amand, 1960), indicated the existence of two distinct types controlled by two alleles at a single autosomal locus. Hb^S was designated as the allele controlling the single, homogeneous or Type I hemoglobin and Hb^d was the allele controlling the diffuse, multibanded, Type II pattern. Neither of the above techniques demonstrated whether dominance or codominance existed in the heterozygote (Popp and St. Amand, 1960).

Petras (1967) reported that by using a modification of Popp and St. Amand's (1960) technique, discernment between homozygous and heterozygous diffuse hemoglobin patterns was possible with careful observation. However, storage of hemolysates for more than two days made such distinction impossible. Even with fresh lysates repetition of some electrophoretic runs was necessary for positive typing.

Popp (1962) reported that the difference in electrophoretic patterns between the two types of mouse hemoglobin

were the result of amino acid differences in their β- chains. The nomenclature for genetic variants of the hemoglobin β-chain was revised by Popp (1965) such that Hbb designated the locus controlling the production of β- chains with Hbb^s and Hbb^d replacing Hb^s and Hb^d, respectively, as the allelic designations. He also suggested that "the locus controlling the β - chains of diffuse hemoglobin is assumed to be compound, probably consisting of two adjacent cistrons", resulting in the production of two or more different β - chains which gives two or more bands on electrophoresis.

In 1962 Morton reported a diffuse hemoglobin variant in which the position of some of the minor bands differed from the normal diffuse pattern. He considered this variant to be controlled by a third allele at the Hbb locus. Morton (1966) subsequently found that several of the minor bands were interconvertible with the major bands upon treatment with mercaptoethanol. As a result, he concluded that at least one trailing band in each diffuse pattern is "a dimer of the whole hemoglobin molecule almost certainly joined by a sulphur bridge".

Riggs (1965) reported the appearance of a higher molecular weight component in diffuse hemoglobin after storage for several weeks. Also, electrophoresis of the diffuse hemoglobin showed a trailing band. Both the trailing band and the higher molecular weight component did not appear after the hemolysate was treated with 2-mercaptoethanol. Riggs concluded that the trailing band observed after

electrophoresis of diffuse hemoglobin was due to the polymerization of the diffuse hemoglobin tetramer to an eight-chain octamer (dimer of Morton's nomenclature), probably by disulphide bridging.

This section describes an electrophoretic technique based on a Tris-maleate-NaOH buffer at pH 7.0 which simplifies the electrophoretic patterns of mouse hemoglobins and so facilitates typing. Homozygous single and diffuse have respectively a single slow and a single fast migrating band. The heterozygote had both a slow and fast band corresponding in migration rate to the slow and fast bands of the parents. Hypotheses, based on experiments with various sulfhydryl reagents, have been proposed in an attempt to explain some electrophoretic patterns.

Materials and Methods

Red blood cell lysates were prepared according to the technique reported by Biddle and Petras (1967). A 14% (w/v) starch-gel was made with hydrolyzed potato starch (Connaught Laboratories, Toronto, Ontario) and a 0.2 M, pH 7.0 Tris-maleate-NaOH buffer (Burstone, 1962).

The buffer was prepared from two stock solutions. Solution A was made by dissolving 32.9 g of maleic anhydride (Fisher) and 48.4 g of Tris (Sigma) in two liters of distilled water. Solution B was 0.2 M NaOH (8 g/liter). To prepare the gel buffer, sufficient solution B was added to the volume of solution A (25 ml. solution A/100 ml. final volume

of gel buffer) to bring the pH to 7.0. The mixture of solution A and solution B was then diluted to the final volume with distilled water. The bridge buffer was made by mixing solution A and B until the hydrogen ion concentration reached pH 7.0. It was then used undiluted. The hot starch-buffer mixture was poured in excess. The excess was removed after the mixture had cooled for one hour.

The lysate was applied to a 4.0 mm x 4.0 mm piece of Whatman #1 filter paper. This was blotted to remove excess hemolysate and then placed in a vertical slot cut across the width of the gel. If the sample rows are made about 3.0 cm apart on a gel 30.0 cm long, 15.0 cm wide, and 0.5 cm thick, theoretically up to 120 samples could be run on a gel. Electrophoresis took place at 4°C with a voltage drop of 5V/cm of gel, until good separation was seen (about 5 hours). After electrophoresis the various sections of the gel were marked for identification. They were then removed from the tray and sliced horizontally into two layers, the top layer being discarded. Typing of the bottom layer could be done at this stage or after staining. Staining was accomplished using a saturated solution amido black in protein wash. The gels were preserved by soaking in protein wash (methanol, acetic acid, water; 5:1:5) for several days and then wrapping in saran wrap.

A correlation study comparing phenotypes obtained with the pH 8.6 borate-metaborate buffer system (Petras, 1967) to phenotypes obtained using the pH 7.0 Tris-maleate-NaOH

system was made using a sample of 108 F_2 offspring randomly chosen from a colony established by mating some C3H to some C57BL/10 inbred mice.

The following reagents, maleic acid (Fisher Scientific, 3.29 g/200 ml. distilled H_2O neutralized with NaOH to pH 7.1), iodoacetate (Sigma, 6.84 mg/ml distilled H_2O neutralized to pH 6.98 with NaOH), iodoacetamide (Sigma, 6.84 mg/ml distilled H_2O, 2-mercaptoethanol (Sigma, 1.7 ml. made up to 100 ml. with distilled H_2O), Tris (Sigma, 4.84 g/200 ml. distilled H_2O), Tris-maleate-NaOH gel buffer at pH 7.0, 0.1 M oxidized glutathione (neutralized with NaOH), and distilled H_2O (as a control reagent) were mixed singly or in combination in equal volume with the single and diffuse hemolysates. The treated lysates were electrophoresed on a 14% starch-gel prepared from a pH 8.6 borate-metaborate buffer (Petras, 1967).

Results

The results of subjecting lysates from animals known to have single (C57BL/10), heterozygous diffuse (C3H x C57BL/10) and homozygous diffuse (C3H) hemoglobins to starch-gel electrophoresis using a pH 8.6 borate buffer system (Petras, 1967) and a Tris-maleate-NaOH buffer system, can be seen in Figure 2 (a) and 2 (b), respectively. With the borate buffer system the single hemoglobin appears as a homogeneous band migrating anodally, the heterozygous-diffuse appears as a broad zone with a band at the anodal end of this zone, and

FIGURE 2 (a): Patterns of mouse hemoglobin after electrophoresis with a pH 8.6 borate-metaborate buffer (Petras, 1967) and staining with amido black.

FIGURE 2 (b): Patterns of mouse hemoglobin after electrophoresis with a pH 7.0 Tris-maleate-NaOH buffer (Petras and Martin, 1969) and staining with amido black.

the homozygous diffuse appears to have a major band migrating at the same rate as the band seen in the single and heterozygous diffuse hemoglobin and a minor band migrating more slowly. With starch-gel electrophoresis in the pH 7.0 Tris-maleate-NaOH buffer system, the single hemoglobin appears as a single cathodal-migrating band, the homozygous diffuse appears as a band migrating anodally, and the heterozygous diffuse has a cathodal and anodal band migrating at the same rate as the cathodal band of single and the anodal band of homozygous diffuse, respectively.

Typing hemolysates of 108 F_2 offspring from the C3H x C57BL/10 colony using the pH 8.6 borate-metaborate and the pH 7.0 Tris-maleate-NaOH buffer systems resulted in a perfect correlation of phenotypes.

Treating C3H (homozygous diffuse) and C57BL/10 (single) hemolysates with neutralized maleic acid, tris, or distilled water resulted in no modification of the C57BL/10 patterns when run on a 14%, pH 8.6 borate-metaborate gel. A C3H lysate, when treated with distilled water or tris, gave the same pattern as seen in Figure 2 (a). Treatment with maleic acid, however, resulted in a faster migration of the C3H hemoglobin causing the hemoglobin to lie more anodally than the distilled water-treated lysate. Iodoacetate was also found to affect the migration of C3H hemoglobin. It gave the hemoglobin a rate of migration intermediate to the faster migration caused by maleate and the normal rate seen in distilled water-treated lysates. Iodoacetamide-treated

C3H hemoglobin was found to migrate at the untreated rate.

C3H and C57BL/10 lysates were treated with equal volumes of distilled water, maleate, a mixture of equal volumes of maleate and iodoacetate, and a mixture of equal volumes of maleate, iodoacetate, and iodoacetamide. The results are shown in Figure 3. None of the treatments had any affect on the migration of C57BL/10 hemoglobin. The maleate-treated C3H hemoglobin migrated anodally as a fast single band. C3H hemoglobin treated with a mixture of maleate and iodoacetate exhibited two bands. Each band corresponded to the hemoglobin pattern obtained by treatment with one of the reagents alone. Treatment with a mixture of maleate, iodoacetate, and iodoacetamide resulted in three bands of C3H hemoglobin, one band with the fast migrating rate, one with the intermediate rate, and one with the normal rate.

Figure 4 shows the electrophoretic patterns obtained after C3H lysates had been reacted with the various reagents and combinations of reagents for thirty minutes and for forty-eight hours. The thirty minute maleate, iodoacetate, or maleate-iodoacetate treated samples possess a band migrating at the normal rate which is absent in samples treated forty-eight hours with the same reagents. The lysate treated with oxidized glutathione appears to have an intermediate migration rate. The remaining samples showed a band or combination of bands similar to those seen in Figure 3.

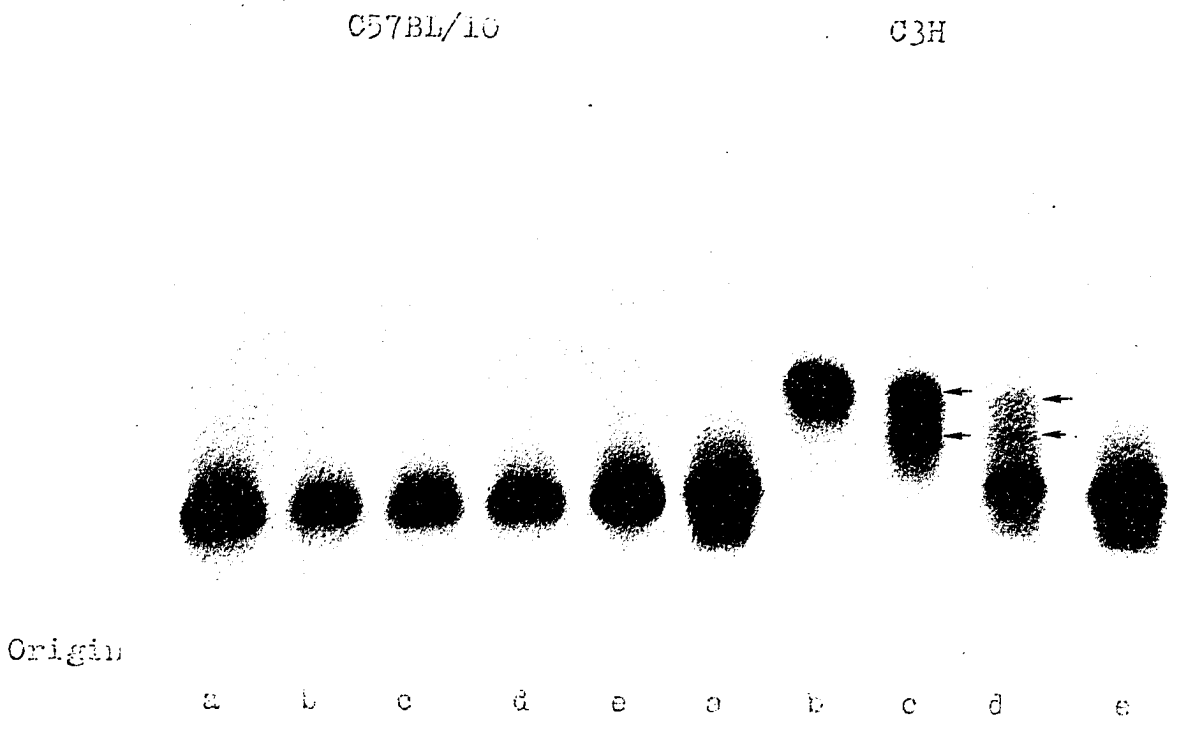

FIGURE 3: Patterns of C57BL/10 and C3H hemoglobins after being diluted 1/1 with water (a, e), maleate (b), maleate and iodoacetate (c), maleate, iodoacetate, and iodoacetamide (d) following electrophoresis in a pH 8.6 borate buffer (Petras, 1967) and staining with amido black.

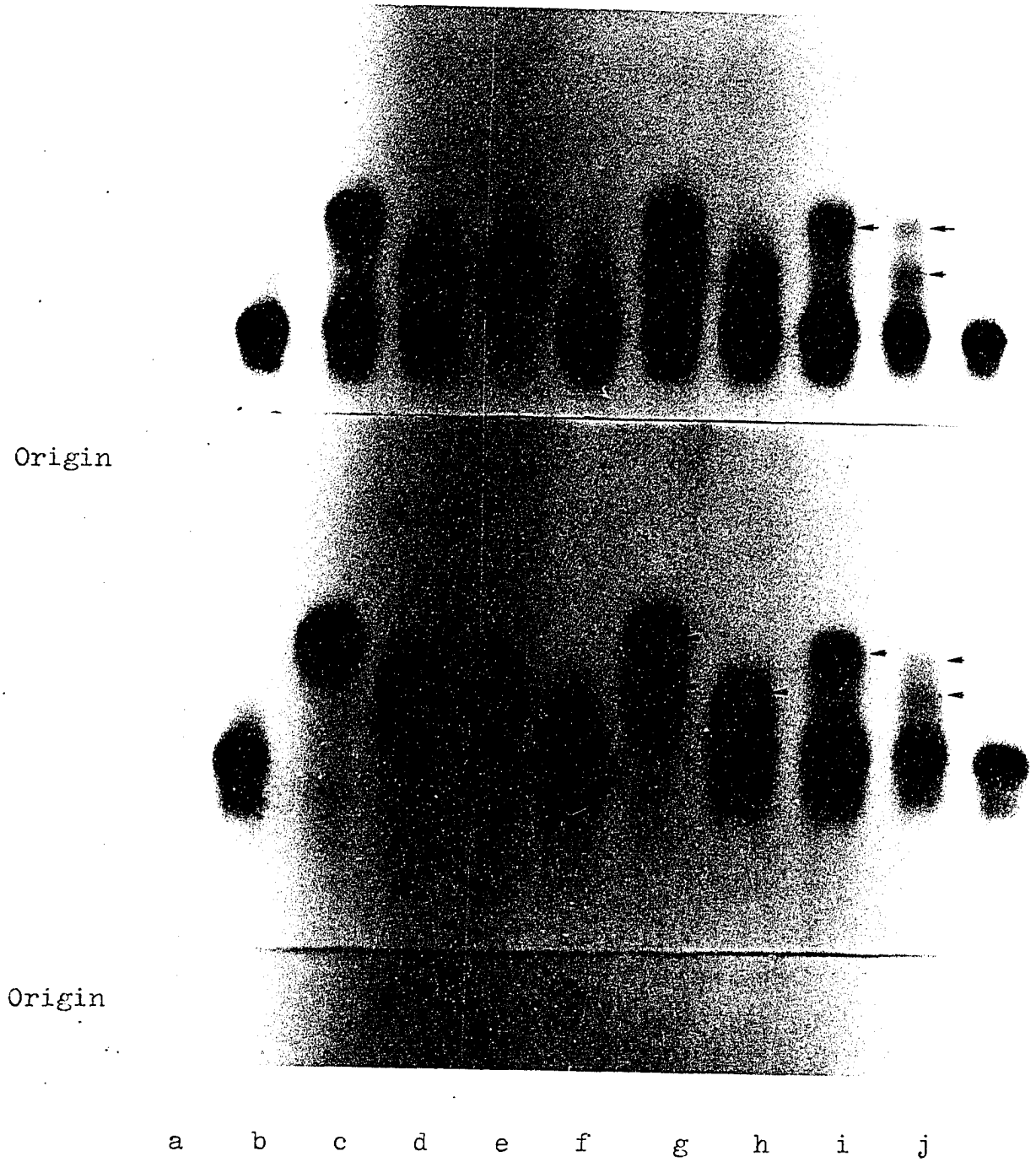

FIGURE 4: Patterns of C3H hemoglobin after 30 minutes (top) and 48 hours (bottom) treatment with water (a, j), maleate (b), iodoacetate (c), glutathione (d), iodoacetamide (e), maleate and iodoacetate (f), iodoacetate and iodoacetamide (g), maleate and iodoacetamide (h), maleate, iodoacetate and iodoacetamide (i), following electrophoresis (Petras, 1967) and staining with amido black.

Discussion

The pH 7.0 Tris-maleate-NaOH buffer system gives reproducible, distinctly resolved, electrophoretic patterns of mouse hemoglobins. Because of the high correlation obtained from the comparison study of hemoglobin phenotypes classified by the pH 7.0 Tris-maleate-NaOH buffer system and the pH 8.6 borate-metaborate buffer, it would seem that both systems are detecting the same genotypes of the Hbb locus.

Riggs (1965) and Morton (1966) have reported that a sulfhydryl group on the β - chain of diffuse hemoglobin is responsible for the presence of the trailing band (a polymer formed by a disulphide bond) and for the interconvertibility of some bands of the diffuse pattern, respectively. Since maleic acid is known to combine with sulfhydryl groups and in doing so adds two negatively charged carboxyl groups at a pH of 7.0 (Webb, 1966), it does not seem unreasonable to assume that some of the maleic acid of the buffer combines with the sulfhydryl groups of the "diffuse" β - chain, thereby altering the net charge of the molecule and also inhibiting polymerization, which would explain the presence of only one band. Since single hemoglobin has no free sulfhydryl group with which maleic acid could bind, the migration is not affected. As a result, the two types of hemoglobin have been separated because of the charge introduced on one by the maleic acid.

As a further test of this hypothesis, both single and "diffuse" hemoglobins were mixed with other sulfhydryl

reagents, including ones which add a single negative charge, iodoacetate and glutathione, and one which would add no charge, iodoacetamide. These reagents were used singly and in various combinations. In no case was the migration rate of single hemoglobin affected, as would be expected if free sulfhydryl groups were absent. The migration of diffuse hemoglobin was affected by all sulfhydryl reagents except iodoacetamide; the exception is expected since iodoacetamide adds no charge.

The effects of iodoacetate or glutathione on the migration rate of diffuse hemoglobin are less than that of maleate treated hemoglobin. Again, this is expected since these reagents have only one negative charge per molecule and as a result, the change in net negative charge of the hemoglobin molecule is half of that found with maleate. Diffuse hemoglobin treated by combinations of these reagents demonstrated several bands. Each band corresponded in migration rate to a single band produced by treatment with one of the components of the mixture. This would indicate that the reagents in the mixture are probably competing for the same reactive site. In all instances where iodoacetamide was part of the reaction mixture, the slowest migrating (indicative of iodoacetamide reaction) band was more intensely stained than any other. This would suggest that more hemoglobin molecules have reacted with iodoacetamide than with the other reagents of the mixture.

When diffuse hemoglobin was mixed with several sulfhydryl

reagents, as many hemoglobin bands appears after electrophoresis as there were differently charged reagents. The absence of any band with an intermediate migration rate (intermediate to the rates observed with two differently charged sulfhydryl reagents) suggests that only one sulfhydryl reagent is bound to one diffuse hemoglobin molecule. Bonaventura and Riggs (1967), however, have reported the presence of two free sulfhydryl groups per diffuse hemoglobin molecule, one on each β - chain. Of the numerous hypothesis which could be postulated in an attempt to explain these conflicting results, the following three appear to be the most likely:

1) The diffuse hemoglobin molecule splits into $2\alpha\beta$ dimers.

This molecular disassociation has been shown to occur in human hemoglobin which has been stored in 0.2 M NaCl (Guidotti, 1967). Each dimer would have one available sulfhydryl group with which it could react. Since there would be only one -SH group per hemoglobin dimer, then only the same number of hemoglobin bands as there are sulfhydryl reagents in a reaction mixture would be observed after electrophoresis. One way of testing this hypothesis would be to establish the molecular weight of the diffuse hemoglobins which form the various bands on the starch gel. This could be done by isolating the hemoglobin bands and chromatographing them in a Sephadex column which has been standardized with proteins of known molecular weights. If the

isolated hemoglobins have a molecular weight of about 63,500 [the normal molecular weight of tetramer hemoglobin, Riggs (1963)], this hypothesis can be disregarded.

2) The same reagent binds to both sulfhydryl groups of the diffuse hemoglobin molecule.

It would seem unlikely that if iodoacetamide could bind to one sulfhydryl group, iodoacetate could not bind to the other, since they differ only by the absence of an amino group in the latter molecule. One way to test this would be to add a known excess amount of the specific sulfhydryl reagent to a known concentration of hemoglobin, and then determine the amount of reagent remaining free in solution after a period of time sufficient for the reaction to take place. The difference between the original amount of the reagent and the amount of the reagent remaining in solution will be the amount reacted with the hemoglobin. If this difference is equal to the concentration of the hemoglobin, then only one molecule of reagent is bound to one molecule of hemoglobin, and the hypothesis can be discarded.

3) The binding of one molecule to one sulfhydryl group alters the environment about the second sulfhydryl group, and prevents a second molecule from binding.

If the two sulfhydryl groups are in close proximity and a charged molecule binds to one, the presence of the charge might repel another molecule and thus prevent it from binding at the second sulfhydryl group.

This could be tested by comparing the amount of charged and uncharged molecules which bind to the hemoglobin. If the hypothesis is valid, the concentration of uncharged molecules binding should be twice that of charged molecules binding. Another aspect of this same hypothesis would involve a change in tertiary structure of the hemoglobin molecule, after one sulfhydryl reagent is bound to one sulfhydryl group. This would result in the second site becoming unavailable for reaction. To test this latter aspect would be most difficult.

In the above three hypotheses, it is assumed there is only one β - chain locus and only one type of β - chain per diffuse hemoglobin molecule. In the last hypothesis, it will be assumed that there are two different hemoglobins with different β - chains, and thus two different β - chain loci. This has already been suggested as a partial explanation for the multibanded diffuse pattern (Popp, 1962; Hutton et.al., 1962). The single band of "diffuse" hemoglobin obtained with the pH 7.0 Tris-maleate-NaOH buffer system suggests that possibly only one type of diffuse molecule is present. However, since two different buffer systems are being used, two different but closely linked loci might be present. It is interesting to note that most typing of mouse hemoglobin has been done at pH 8.6. At this pH sulfhydryl groups begin to dissociate (Webb, 1966). Thus, the extra band may be an artifact due to the pH of the

electrophoretic buffer system. To determine whether two different β - chains are present in diffuse hemoglobin, it would be necessary to know the amino acid sequence of the β - chain from this molecule. Such data are not yet available.

Conclusions

The results strongly support the probability that the pH 7.0 Tris-maleate-NaOH buffer and the pH 8.6 borate-metaborate buffer are detecting the same alleles of the Hbb locus. The increased resolution of the hemoglobin phenotypes obtained with the Tris-maleate-NaOH buffer seems to be due to the presence of a free sulfhydryl group on the diffuse hemoglobin molecule which permits the binding of maleate and other sulfhydryl reagents. The binding of maleate would increase the net negative charge of the molecule, giving it a faster migration rate than the single hemoglobin during electrophoresis. The sulfhydryl group must also be present in some heterozygote hemoglobin molecules, for the heterozygote phenotype has two distinct components, each having a migration rate comparable to the parental phenotypes. This latter point would indicate that the two hemoglobin alleles Hbb^d, Hbb^s are probably codominant.

Several hypotheses have been presented as explanations for the results obtained from the electrophoresis of treated diffuse hemoglobin. Recent work by Hutton (1969) suggests that the binding of one charged molecule to the diffuse

hemoglobin tetramer may prevent another sulfhydryl reagent from binding at the second sulfhydryl group. This would explain why it appears that only one reagent binds per hemoglobin molecule, when it has been shown (Bonaventura and Riggs, 1967) that two sites are available.

CHAPTER IV

TWO ERYTHROCYTIC LACTATE DEHYDROGENASE VARIANTS

After starch-gel electrophoresis and appropriate staining, homogenates of different tissues of the house mouse, Mus musculus, yield characteristic lactate dehydrogenase (LDH) patterns. For example, heart tissue yields only the fastest migrating zones of enzyme activity, liver and muscle exhibit only the slowest migrating zones, while kidney tissue gives five zones (including the fastest and slowest migrating) of LDH activity (Markert and Ursprung, 1962). The most anodal zone of activity has been found to be due to an LDH which is composed of four identical polypeptides which are referred to as B polypeptides. The slowest migrating zone of LDH activity is also composed of four identical polypeptides which are different from B and are designated A polypeptides (Markert, 1963). An in vitro dissociation and recombination of an equal mixture of pure A and B polypeptides resulted in five electrophoretically-separable, enzymatically-active zones (Markert, 1963). The artificially produced hybrid zones were electrophoretically identical to those intermediate zones found in some tissue homogenates. Thus, random recombination of the two electrophoretically distinct subunits to form all possible tetramer species of LDH was suggested as an explanation of the five zones of activity (Markert, 1963).

Shaw and Barto (1963) found the first inherited variant of mammalian lactate dehydrogenase in Peromyscus maniculatus, the deer mouse. The electrophoretic patterns and breeding data were consistent with Markert's hypothesis and the variant discovered was the result of a mutant allele at the locus controlling the production of the B polypeptide in Peromyscus. It was suggested that locus A and locus B be designated as the loci which control the primary structure of the A and B polypeptides, respectively. Subsequently, studies of human LDH revealed both A-subunit (Nance, et al., 1963; Kraus and Neely, 1964) and B-subunit (Boyer, et al., 1963; Kraus and Neely, 1964; Vesell, 1965) variants. To date no LDH structural loci have been reported in Mus musculus. Riles (1965) has described the occurrence of an LDH zone in erythrocytic lysates from CBA mice, which was not evident in C57BL/10 mice. She attributed control of this difference to "one gene". Recently, Shows and Ruddle (1968) have found a similar difference in other inbred strains of mice. They did not consider it identical to the one described by Riles.

In this report, two variant erythrocytic LDH patterns will be described: one is the presence or absence of the band (LDH IV) next to the slowest; the other involves the slowest band (LDH V) and is found between two inbred strains of Mus musculus. The former is attributable to a regulatory locus which controls the synthesis of polypeptide B in the erythrocytes, and the latter to factors which affect the

electrophoretic migration of polypeptide A.

Materials and Methods

Matings between some wild and C57BL/10 mice were established to test for the mode of inheritance of the variant involving LDH IV. The animals used to test for the mode of inheritance of LDH V were from a colony of C3H x C57BL/10 inbred mice.

Erythrocytic lysates were prepared according to the procedure described by Biddle and Petras (1967).

Skeletal muscle, kidney and heart were removed from mice killed by a blow on the head. The organs were washed thoroughly in cold, physiological saline. The tissues were then homogenized with equal volumes of distilled water in a glass homogenizer. After freezing and thawing the homogenates three times, the samples were centrifuged at 27,000 x g for one hour in a refrigerated centrifuge (4°C). The clear supernatant was removed and stored at 2-3°C.

The electrophoretic apparatus and method of preparing a gel are basically those described by Biddle and Petras (1967). To demonstrate the presence of LDH IV, a sixteen per cent starch-gel and Kristjannson's (1963) discontinuous buffer system were used. A modification of the buffer system reported by Ruddle, Shows and Roderick (1968) was employed in the study of LDH V. A stock solution of buffer was prepared by mixing 0.5 M boric acid (Fisher Scientific), 0.02 M EDTA (disodium salt of ethylenediaminotetraacetic acid,

Sigma) and sufficient primary standard, crystalline tris (trihydroxyaminomethane, Sigma) to bring the solution to pH 7.0. The dilutions reported by Shows, Ruddle and Roderick (1/20 for the 14% starch-gel, 1/5 for the cathode, and 1/7 for the anode) were used.

For determination of LDH IV patterns, erythrocyte lysates and tissue homogenates less than one week old were used. Erythrocyte lysates exactly three weeks old were used for the detection of the LDH V variant.

Both systems were run at 4°C using a potential difference of 10 V/cm. For the detection of LDH IV, electrophoresis was carried out until the borate band migrated 10 cm., a period of about 3½ hours. Electrophoresis for seventeen hours was necessary to separate the LDH V variants. After electrophoresis was complete, the gels were removed from the mold and sliced horizontally into two layers. The bottom layer was stained for lactate dehydrogenase activity.

The procedure used for staining was a modification of that reported by Markert and Faulhaber (1965). The stain solution was composed of 200 ml., 0.1 M Tris-HCl buffer at pH 8.0., 20 mg. nitroblue tetrazolium (Sigma), 15 mg. phenazine methosulphate (Sigma); 40 mg. nicotinamide adenine dinucleotide (Sigma); and 4 ml. sodium lactate syrup (Fisher). The stain mixture was poured over the gel and both were incubated at 37°C in the dark until staining reached an optimum (about 1½ hours).

For further characterization of the LDH V variant, some

erythrocyctic lysates and some tissue homogenates were mixed 1:1 with 0.1 M oxidized glutathione (neutralized with NaOH).

Results

Two electrophoretic patterns have been observed with the technique used for the LDH IV variant. Both patterns contain a slow, anodally migrating zone of LDH activity corresponding to LDH V of tissue homogenates (Figure 5). Found in the erythrocytes of many wild mice was a more rapidly migrating anodal zone of activity comparable to LDH IV of tissue homogenates (Figure 5). Lysates from C3H, C57BL/10, and CBA/J inbred strains of mice lacked this faster component. Matings between wild mice possessing the erythrocytic LDH IV and C57BL/10 were established. A summary of the breeding data is presented in Table 3. These results are consistent with a one-locus, two-alleles mode of inheritance. LDH patterns of tissue homogenates (kidney, liver and muscle) from animals possessing LDH IV in the erythrocytes and lacking erythrocytic LDH IV appeared identical.

No LDH V variant was observed when fresh lysates were examined after appropriate electrophoresis and staining. Three LDH V patterns were observed after these lysates had been stored for three weeks and were subjected to electrophoresis (Figure 6). The C57BL/10 patterns contained one to three anodal-migrating subbands, whereas the C3H pattern contained up to four more slowly migrating subbands of LDH activity, with the most anodal and cathodal subbands,

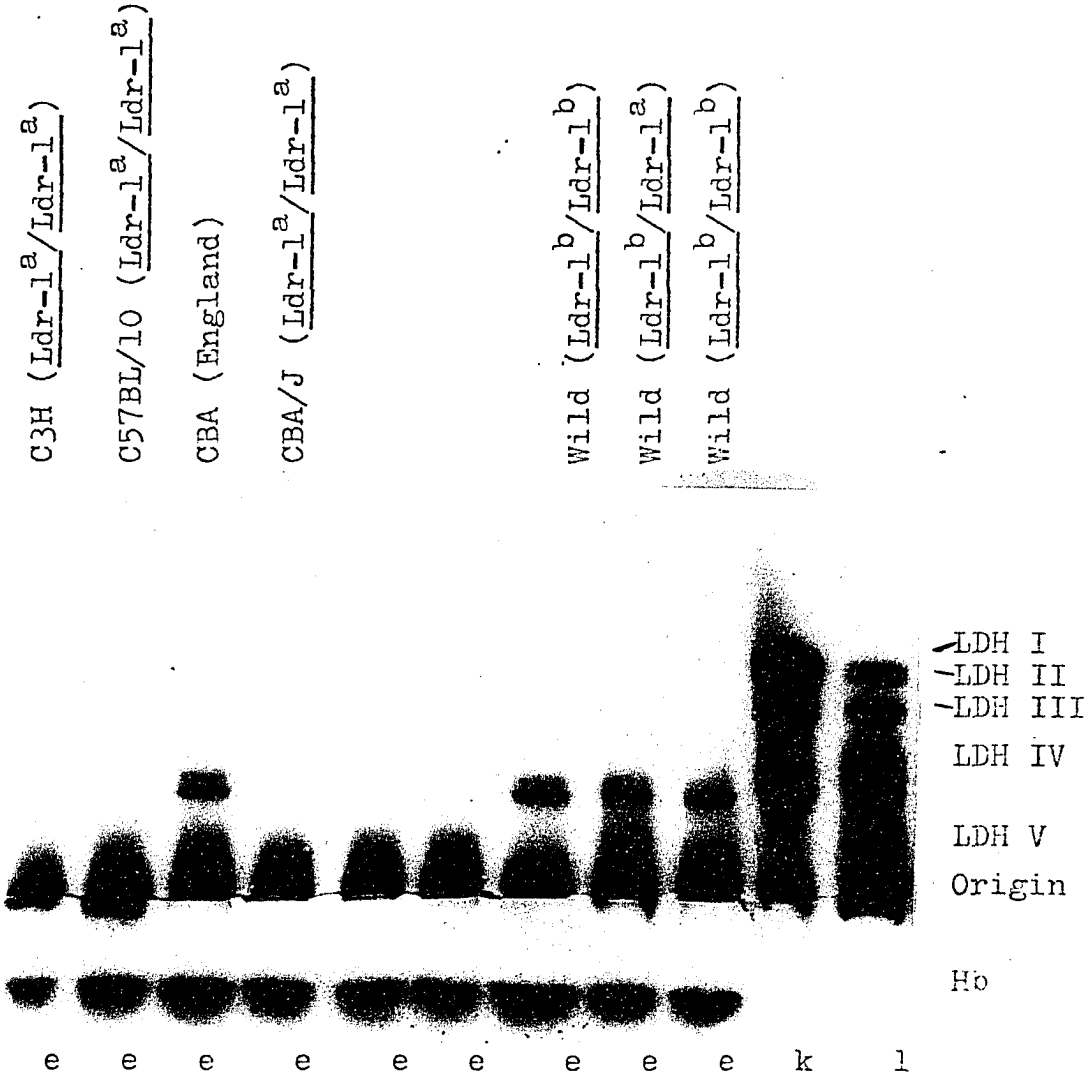

FIGURE 5: Patterns of lactate dehydrogenase isozymes from erythrocytes (e), kidney (k), and liver (l) tissue after electrophoresis (Kristjannson, 1963) and staining (Markert and Faulhaber, 1965).

TABLE 3

Breeding Data from Matings Set Up to Test for the Mode of Inheritance of the LDH IV Variant

Matings or Inbred	Phenotypes		X^2	P
	LDH IV Zone Present	LDH IV Zone Absent		
C57BL x C57BL	0	20 *	-	-
C3H x C3H	0	20 *	-	-
Wild x C57BL	31	0	-	-
F_1 x F_1	79	22	0.557	>0.30
F_1 x C57BL	52	59	0.442	>0.50

* All C57BL and C3H mice typed were of this phenotype.

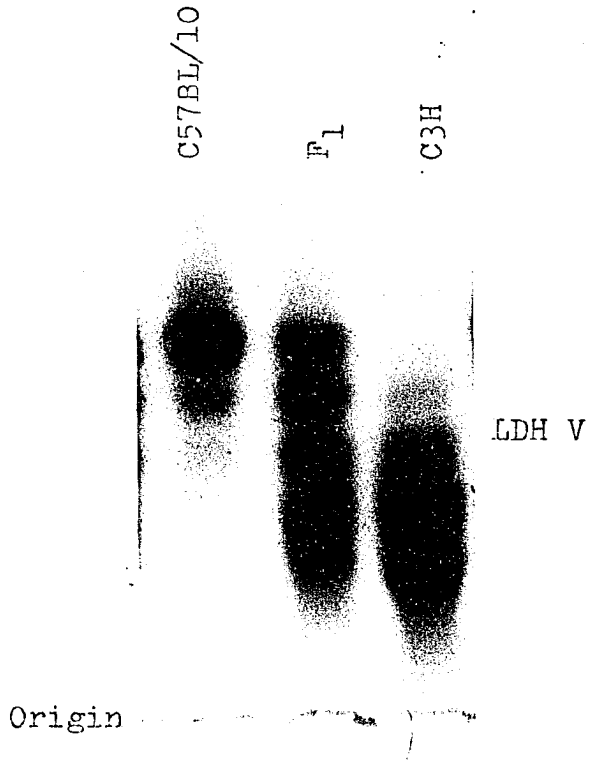

FIGURE 6: Patterns of the LDH V variant after electrophoresis (Ruddle, Shows and Roderick, 1968) and staining (Markert and Faulhaber, 1965).

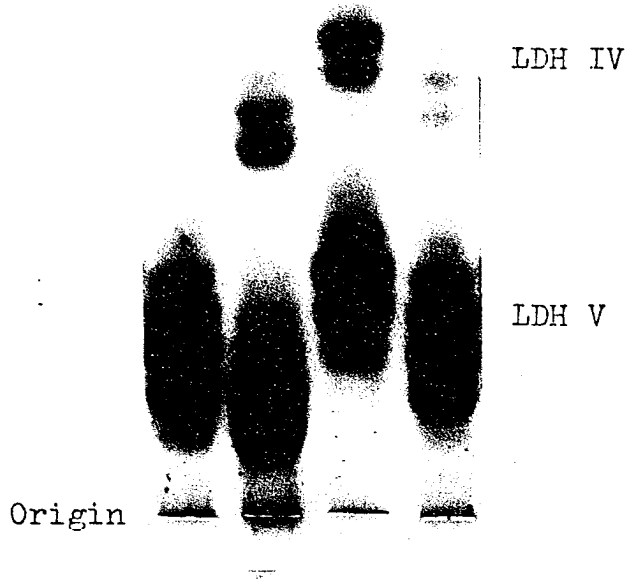

FIGURE 7: Patterns of LDH from lysates stored for three weeks after electrophoresis (Ruddle, Shows and Roderick, 1968) and staining (Markert and Faulhaber, 1965).

respectively, staining with the greatest intensity. The F_1 (C3H x C57BL/10) pattern consisted of five anodal migrating subbands, with the fastest band corresponding to the most anodal component of the C57BL/10 pattern, and the slowest-migrating subband comparable to the most cathodal C3H subband. The F_1 zone of activity was comparable to LDH V of tissue homogenates. Figure 7 shows that after three weeks of storage, the migration of LDH IV, when this zone is present, is also affected and in the same manner as LDH V. A mating study involving C3H and C57BL/10 mice suggests that the LDH V variants are controlled by two alleles at a single locus (Table 4).

Linkage studies revealed that the locus controlling the appearance of LDH IV was not sex-linked, nor was it linked to Hbb, Ea-1, Es-1, Es-3, Es-5, or the agouti locus. The locus controlling LDH V appears to be closely linked to Hbb on linkage group 1. The test for association of phenotypes is shown in Table 5.

An examination of several wild populations showed that of the 291 animals typed for LDH IV patterns, 233 had LDH IV in the erythrocytes and 58 did not. In the 181 mice typed for LDH V, 66 had the fast pattern, 55 the slow, and 60 the intermediate pattern.

Discussion of Genetic Control

LDH IV variant.

The breeding data appear consistent with the hypothesis

TABLE 4

Breeding Data from Matings Set Up to Test
for the Mode of Inheritance of the LDH V Patterns

Matings	Phenotypes			X^2	P
	LDH V Bands	LDH V Bands	LDH V Bands		
C57BL x C57BL	10	0	0	-	-
C3H x C3H	0	0	10	-	-
C57BL x C3H	0	19	0	-	-
F_1 x F_1	22	31	15	1.97	>0.30
F_1 x C3H	0	6	7	0.078	>0.70
F_1 x C57BL	11	12	0	0.044	>0.80

TABLE 5

Contingency Table Describing the Association of LDH V and Hemoglobin Phenotypes from C3H and C57BL/10 Mice

		Hemoglobin Phenotypes	
		Diffuse and Heterozygote	Single
LDH V Phenotypes	Fast	0 (14.32)*	22 (7.68)
	Intermediate and Slow	41 (26.68)	0 (14.32)

X^2 Analyses:	D.F.	X^2	P
Segregation at LDH V Controlling Locus	2	2.22	>0.30
Segregation at Hbb	2	2.38	>0.30
Association of Phenotypes	1	63.03	<0.001

Degree of Association:	X^2	n	X^2/n
	63.03	63	1.0

Conclusion: very close association of phenotypes.

* Expected values are in brackets.

that the isozyme four (LDH IV) variant is controlled by two alleles at an autosomal locus. The presence of the anodal component is found in high frequency (0.80) in wild populations, but it is absent in the three inbred strains (C3H, CBA/J, C57BL/10) examined. Because of the similarity in electrophoretic patterns, linkage results and population data of LDH IV and the LDH controlled by locus Ldr-1 reported by Shows and Ruddle (1968), there is considerable likelihood that the locus being described here is identical to Ldr-1.

Ldr-1 appears to be the first regulatory locus found in the house mouse. During the studies of the variant several theories as to the mode of action of the locus have arisen. The first is that the locus may be producing something which blocks the production of the LDH A polypeptide. The second theory is that the gene may function early or late in red cell development. If the gene began regulating the production of the A polypeptide early in red cell growth, no A polypeptide would be present. If it functioned only during the later stages of red cell development, a limited amount of the A polypeptide could be present and LDH IV could be observed. In both cases, it is assumed that the B polypeptide is produced first during development and the A polypeptide produced later in growth. This has been reported by Markert and Ursprung (1962) for other organs of the house mouse.

Shows and Ruddle (1968) have suggested that since

isozyme four was not detected in the erythrocytes of CBA/J mice, the Ldr-1 locus cannot be the same gene reported by Riles (1965). Recently, we have obtained from the University of London, London, England, members of the same CBA strain of mice which Riles had investigated. After electrophoresis and staining, these mice were found to possess an LDH zone of activity that was similar to LDH IV. Matings to test for identity of Ldr-1 and the locus controlling the LDH zone found in Riles' CBA mice are to be made.

LDH V variant.

From the results summarized in Table 4, it appears that the LDH V variant is controlled by two alleles at a single autosomal locus. The results given in Table 5 suggest that the locus controlling the LDH V variants is closely linked to Hbb. Studies of wild populations of mice indicate that the locus controlling the LDH V variants is polymorphic, with the allele responsible for the faster migrating proteins having a frequency of 0.53 and the allele responsible for the slower migrating proteins having a frequency of 0.47.

Additional Experiments and Conclusions

After reaching the above conclusions regarding the LDH V variants, two further observations were made almost simultaneously. The first was that storage of lysates for three weeks altered both the LDH V pattern of C57BL/10 mice, and the hemoglobin pattern of C3H mice such that an anodal

migrating band of hemoglobin became evident. Second, Lewis, et al. (1967) referred to an increase in the concentration of oxidized glutathione in human red cell lysates which, in some cases, affected the electrophoretic migration of a particular peptidase. Consequently, fresh C3H and C57BL/10 mouse hemolysates were treated with equal volumes of 0.1 M neutralized, oxidized glutathione. The results are seen in Figure 8.

Fresh, untreated C3H and C57BL/10 lysates have similar LDH and hemoglobin patterns when run with the electrophoretic technique described by Ruddle, Shows, and Roderick (1968). Hemoglobin from a C57BL/10 lysate aged three weeks has the same migration rate as hemoglobin from a fresh lysate. However, the LDH V pattern of stored C57BL/10 hemolysates has an increased migration rate. C3H lysates aged three weeks have two hemoglobin bands, one with a normal migration rate, the other with an increased migration rate. The C3H LDH V has not changed migration rate after three weeks storage. Fresh C3H and C57BL/10 lysates, treated with oxidized glutathione, have LDH patterns similar to the aged C57BL/10 pattern (that is, with an increased migration rate), and the C3H hemoglobin migrates as a single band corresponding in migration rate to the anodal band found in three week old C3H lysates. The migration rate of C57BL/10 hemoglobin was not affected by treatment with oxidized glutathione.

Figure 9 summarizes a hypothesis which represents a possible explanation for these observation. This hypothesis

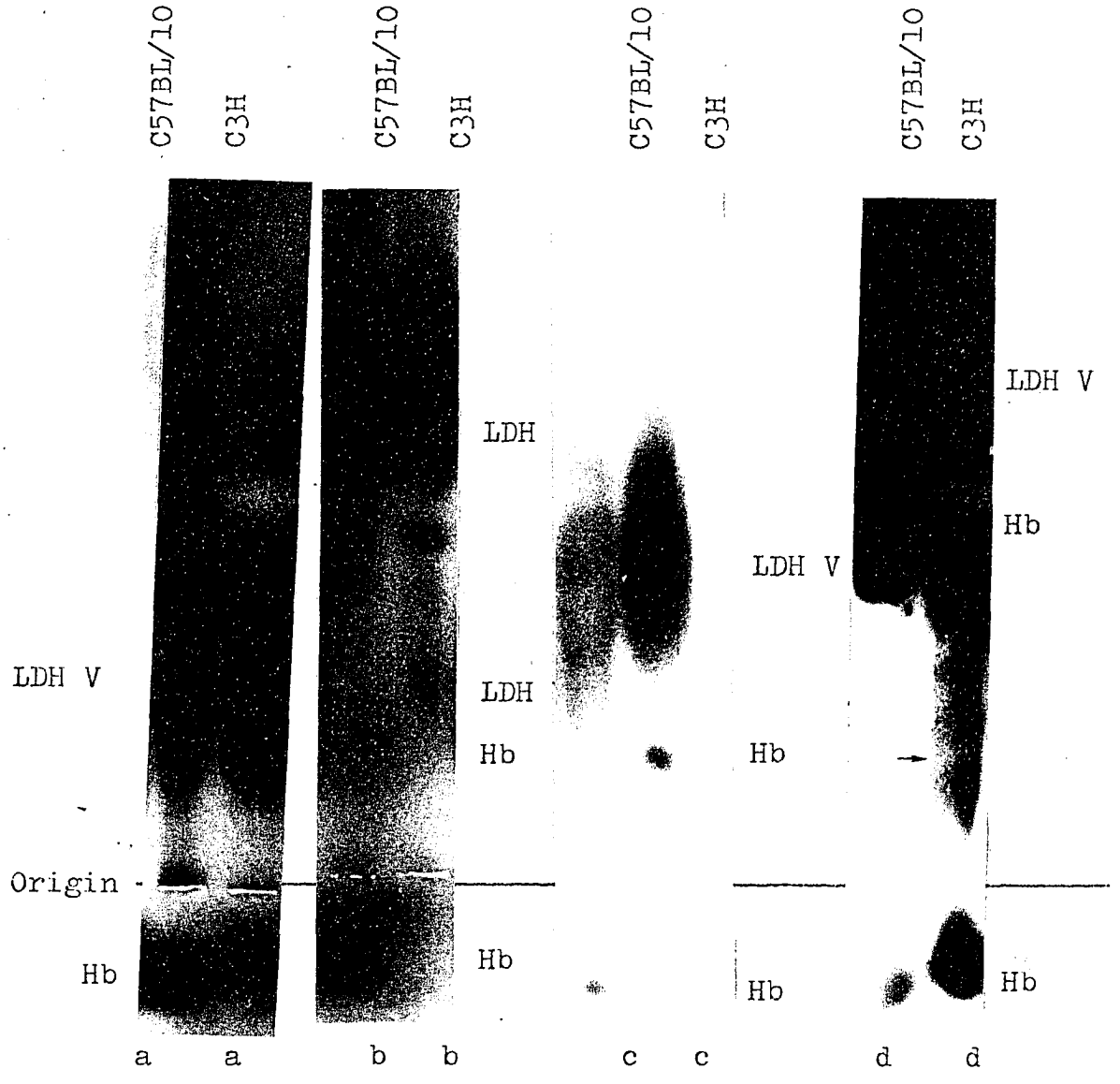

FIGURE 8: Hemoglobin and LDH patterns of (a) fresh lysates, (b) three week old lysates, (c) fresh lysates treated with oxidized glutathione, and (d) fresh lysates treated with maleate from C3H and C57BL/10 mice after electrophoresis (Ruddle, Shows and Roderick, 1968) and staining (Markert and Faulhaber, 1965).

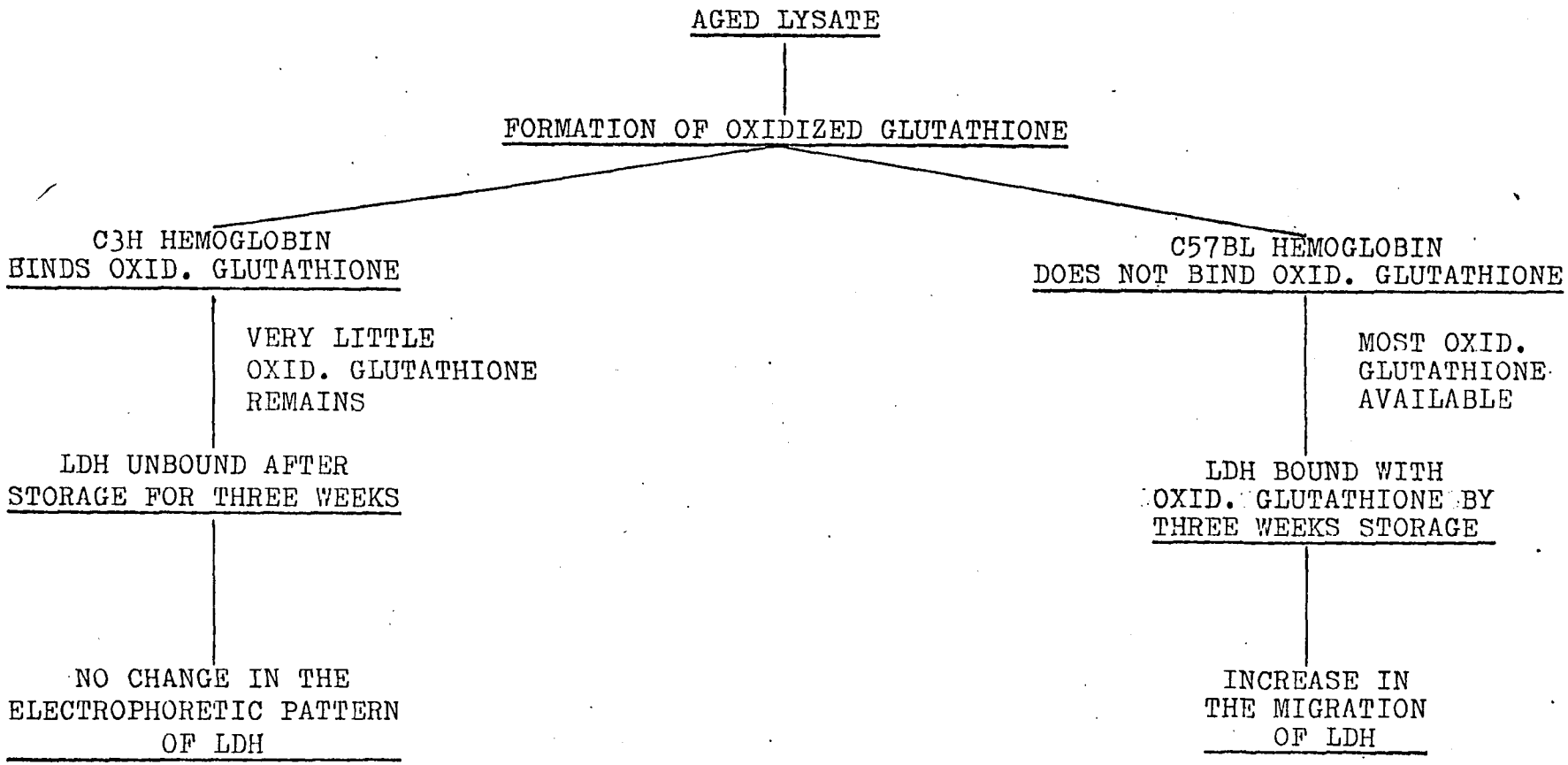

FIGURE 9: Hypothesis to explain the LDH V electrophoretic patterns.

postulates that oxidized glutathione or a similar compound which accumulates in red cell lysates on storage is responsible for the altered migration rate of the LDH and hemoglobin molecules. This accumulating compound first binds with hemoglobin (since it is present in high concentration) if an appropriate site is available and the excess then binds with LDH or some other protein with appropriate sites. In lysates where the accumulation of the reacting compound is negligible if the appropriate hemoglobin is available, no effect on LDH V is observed. In C57BL/10 lysates, where the appropriate hemoglobin is not available, the concentration of the compound reaches a level where it affects the LDH much earlier and the electrophoretic migration of LDH V is altered after three weeks of storage.

Preliminary work with maleate shows that this compound also increases the migration rate of LDH V. This finding is consistent with the hypothesis that the storage product could be bound to the LDH molecule through a disulphide bond.

From the results summarized in Table 4, it appeared that the LDH V variant found between C3H and C57BL/10 mice was controlled by two alleles at one locus. However, it now seems more probable that these variants reflect the presence of a storage product in the lysate, the concentration of which appears to be controlled to a considerable extent by the type of hemoglobin molecule present in the lysate. From Table 5, it can be seen that there is very close

association between the homozygous single hemoglobin and the fast LDH V band. In fact, the degree of association of traits compared in this Table is 1.0. The hemoglobin and LDH V phenotypes of wild mice which are being compared in Table 6 are significantly associated. The degree of association for these phenotypes is 0.16. Thus, the degree of association of the same LDH V and hemoglobin phenotypes is much less among wild animals than between C3H and C57BL/10 mice. This would suggest that there may be a mechanism other than the hemoglobin molecule controlling the concentration of the storage product in erythrocytic lysates of wild mice.

Summary

Two erythrocytic LDH variants have been found. One, involving the presence and absence of the next to the slowest isozyme, is controlled by a regulatory locus which may be similar to Ldr-1 (Shows and Ruddle, 1968). The second appears to be indirectly controlled by the Hbb locus and may be attributed directly to the accumulation of storage product in the lysates. Ldr-1 is polymorphic in natural populations.

TABLE 6

Contingency Table Describing the Association of LDH V and Hemoglobin Phenotypes from Wild Mice Trapped in 1968

		Hemoglobin Phenotypes	
		Diffuse and Heterozygote	Single
LDH V Phenotypes	Fast	0 (14.22)*	66 (51.78)
	Intermediate and Slow	39 (24.78)	76 (90.22)

X^2 Analyses:	D.F.	X^2	P
Association of Phenotypes	1	28.53	<0.001

Degree of Association:	X^2	n	X^2/n
	28.53	181	0.16

Conclusion: there is a significant association of phenotypes.

* Expected values are in the brackets.

CHAPTER V

GENERAL DISCUSSION

As mentioned in the INTRODUCTION, there are four possible causes for proteins controlled by alleles at a single locus to differ in their electrophoretic mobility. The first and simplest reason is a difference in base sequence of the cistron which, at least in some cases, would result in proteins having amino acids of different charges. More than likely this accounts for the separability of the Es-3 controlled esterases. Although there is no direct evidence, it is not unreasonable to assume that if a mutation occurred at a codon which coded for a neutral amino acid in the Es-3b esterase, an acidic or basic amino acid may be produced. This could account for the mobility of Es-3d and Es-3c, respectively. A similar explanation has been reported for the electrophoretic differences found in hemoglobins A, C, and S from humans. The only difference between these hemoglobins was a change in an amino acid of one particular tryptic peptide. The following amino acid sequences are from this peptide (Chernoff, 1961):

Hgb A: Val-His-Leu-Thr-Pro-Glu-Glu-Lys↑

Hgb S: Val-His-Leu-Thr-Pro-Val-Glu-Lys↑

Hgb C: Val-His-Leu-Thr-Pro-Lys-Glu-Lys↑

↑- points of tryptic cleavage

Hemoglobin A moves slightly faster than hemoglobin S during

electrophoresis and hemoglobin C moves equally slower, migrating behind hemoglobin S (Rucknagel, 1968). Since in the electrophoretic patterns of the mouse red blood cell esterases, Es-3b, Es-3c, and Es-3d, and the human hemoglobins Hgb A, Hgb C, and Hgb S, one protein (Es-3b, or Hgb S) is found to migrate midway between a faster moving protein (Es-3d, or Hgb A) and a slower moving protein (Es-3c, or Hgb C), all of which are controlled by alleles at one locus, it does not seem unreasonable to suggest that the causes of both the esterase and hemoglobin electrophoretic patterns may be similar.

The second reason for differences in the electrophoretic migration rate between proteins controlled by alleles at the same locus is the artificial induction of charge differences by means of reagents which react specifically with a site on one molecule, which is absent on the second molecule. There is considerable evidence (Riggs, 1965; Morton, 1966) which suggests the presence of a reactive sulfhydryl group on the β- chains of diffuse hemoglobin and its absence in single hemoglobin β- chains. Thus, if these two types of hemoglobin are mixed with reagents that are known to bind with sulfhydryl groups and if these have a net charge at the pH of the reaction, a difference in charge between the proteins could be artificially induced. This appears to be occurring during electrophoresis of mouse hemoglobin when the pH 7.0 Tris-maleate-NaOH buffer system is used. Here, then, the buffer system is capable of

resolving proteins which differ only slightly, the difference being due to the number of sulfhydryl groups. Since these groups possess no charge at physiological pH's, such variation would not normally be detected.

The third cause of the difference in charge between proteins controlled by alleles at the same locus, is the combining of the proteins with a lysate storage product, the concentration of which is controlled by the product of another locus. It has been seen that changes in migration rates of erythrocytic LDH V from C3H and C57BL/10 lysates occurs after storage for three weeks. The concentration of oxidized glutathione has been stated to increase on storage of red blood cell lysates (Lewis, et al., 1967). Oxidized glutathione is also known to react readily with available sulfhydryl groups (Webb, 1966) and change the net charge by minus one. However, the level of oxidized glutathione may vary from one lysate to the next, due to presence in one lysate of a very reactive glutathione reductase (Long, , 1967) or of a protein present in high concentration (such as diffuse hemoglobin) which is capable of binding oxidized glutathione and thus not permitting it to react with the sulfhydryl groups of other proteins. This might explain why the LDH V from C57BL/10 mice [which have a hemoglobin lacking free sulfhydryl groups (Riggs, 1965)] increases in migration rate, and why the migration rate of C3H LDH V does not change.

The fourth reason for proteins controlled by alleles

at one locus to differ in charge, is the absence of a subunit which possesses a charge different from another subunit. Lactate dehydrogenase is formed as a tetramer of two randomly associated, differently charged polypeptide subunits. The A polypeptide has the more positive charge, with the B subunit having the more negative, giving the A homotetramer the slowest migration and the B homotetramer the fastest under most electrophoretic conditions. If the production of one of these polypeptides was regulated or blocked, only isozymes which are composed mainly of the freely produced polypeptide will be obtained. This seems to be the explanation for the electrophoretic patterns obtained when assaying for the Ldr-1 phenotypes. The difference in charge which permits separation of LDH IV from LDH V is the presence of the B polypeptide in LDH IV. Thus, if the production of the B polypeptide is blocked completely, all isozymes except LDH V are absent. In this example, then, the absence of an electrophoretically separable enzyme (i.e., LDH IV) may be due to the absence of an entire gene product (i.e., the B polypeptide). Closely linked regulator genes may also explain the "silent gene"; that is, the inherited absence of any detectable enzymatic activity [e.g., the products of the genotypes $Es\text{-}2^{a}/Es\text{-}2^{a}$ (Petras, 1963), $Es\text{-}3^{a}/Es\text{-}3^{a}$ (Ruddle and Roderick, 1965)].

Thus, electrophoretic differences between proteins controlled by alleles at the same locus may be due to (a) differences in charged amino acids, (b) the artificial induction

of charge difference due to different numbers of sulfhydryl groups on the proteins, (c) the combining of the protein with a charged storage product, the concentration of which may vary from one sample to another, and (d) the absence of a differently charged polypeptide subunit.

Finally, two general points concerning electrophoretic studies of proteins should be made:

(1) With the Tris-maleate-NaOH buffer system, it is now possible to detect differences in proteins due to differences in the number of free sulfhydryl groups. Thus, more protein variants may be detected even if there is no difference in charge at a particular pH, as long as a sulfhydryl reagent can be attached to a free sulfhydryl group on the protein molecule.

(2) Consideration must be given to possible molecular interactions during storage of a lysate or homogenate. Otherwise, loci may be reported which are nothing more than reflections of the products of other loci. In finding these latter relationships breeding data, and especially linkage date must be considered. This last possibility, as Shaw (1965) pointed out some time ago, when he stated, "An additional advantage of this approach...is that since a mixture of many hundreds or thousands of different enzymes is present in the gel, unexpected relationships may be uncovered, which otherwise would never have been looked for", may lead to some exciting studies.

CHAPTER VI

SUMMARY

1. Another allele, *Es-3*^{d}, at the *Es-3* locus, has been found in some wild mice. The ability to separate the three esterases Es-3b, Es-3c, and Es-3d, electrophoretically is believed to be due to an amino acid substitution at the same position in the three proteins. Three of the *Es-3* alleles, *Es-3*^{b}, *Es-3*^{c}, *Es-3*^{d}, have been found in wild populations. From the present studies, no one allelic product has any visible advantage.

2. An improved resolution of mouse hemoglobins obtained by using a pH 7.0 Tris-maleate-NaOH buffer, appears to be due to the binding of maleate to a -SH group found on diffuse hemoglobin and absent in single hemoglobin. The binding of maleate to the diffuse hemoglobin alone would induce a charge difference between the two hemoglobins. Several hypotheses have been presented which may help to explain some of the electrophoretic patterns of diffuse hemoglobin.

3. A regulatory locus controlling the presence and absence of LDH IV has been found in the red blood cells of many wild mice. It is probably the *Ldr-1* locus reported by Shows and Ruddle (1968) which in turn appears identical to the locus described by Riles (1965).

4. Another variant of LDH involving isozyme five has

been found between C3H and C57BL/10 mice after storage of their erythrocytic lysates for three weeks. It was found that oxidized glutathione causes a similar variation in fresh lysates. A hypothesis which may explain the red blood cell isozyme five variants has been presented.

5. The use of the Tris-maleate-NaOH buffer system can be extended to detect any proteins which differ only in the number of free sulfhydryl groups that each possesses.

6. Consideration must be given to possible reactions that could occur when red blood cell lysates and/or tissue homogenates are stored for a period of time. Electrophoretic variants which appear after storage of the samples may only be reflecting the products of some other locus.

7. The proteins which were studied showed differences in charge which are believed due to (a) amino acid substitutions at one site of the protein, (b) substitution of one amino acid by another which itself does not possess a charge at most physiological pH's, but is quite reactive with sulphur containing compounds and other reagents, (c) the gene-regulated absence of a polypeptide subunit, or (d) interaction between the protein and a charged storage product.

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