*GENETIC EVIDENCE FOR THE SUBUNIT STRUCTURE OF LACTATE DEHYDROGENASE ISOZYMES**

BY CHARLES R. SHAW AND ELIZABETH BARTO

HAWTHORN CENTER, NORTHVILLE, MICHIGAN, AND MAMMALIAN GENETICS CENTER, DEPARTMENT OF ZOOLOGY, UNIVERSITY OF MICHIGAN

Communicated by B. H. Willier, June 17, 1963

The lactate dehydrogenases commonly occur in from one to five molecular forms (isozymes) in the various tissues of an animal species.[1] By means of zone electrophoresis, characteristic patterns of these isozymes may be demonstrated in each tissue. In adult animals, most tissues contain all five isozymes but in different proportions. Usually, heart muscle exhibits principally the first, or most negatively-charged, isozyme (LDH-1), while skeletal muscle and liver contain chiefly the most positively-charged (LDH-5).

Appella and Markert[2] treated crystalline lactate dehydrogenase from beef heart with 5 *M* guanidine-HCl. This reagent dissociates the LDH molecule into four subunits of equal molecular weight, as determined by sedimentation studies. These subunits, or polypeptide chains, were shown to exist in two different electrophoretic varieties which Markert[3] has designated as subunits A and B. Moreover, he theorized that the five isozymes are the five different tetramers that would be obtained by associating these two subunits in all possible combinations of four.[2, 3]

Cahn *et al.*,[4] using chicken LDH, showed that the two "pure" tetramers (LDH-1 and -5) are immunologically distinct and, further, that the three intermediate isozymes (LDH-2, -3, and -4) are cross-reactive with both pure types. Thus, these intermediate isozymes presumably contain both kinds of subunits. Recently, Markert[5] dissociated LDH-1 and LDH-5 into subunits by freezing in 1 *M* NaCl; after thawing, the subunits recombined at random in groups of four to yield all five isozymes in approximately the expected ratio of 1:4:6:4:1. The postulated subunit formulae for the LDH isozymes makes these relationships clear: LDH-1 = BBBB, LDH-2 = BBBA, LDH-3 = BBAA, LDH-4 = BAAA, LDH-5 = AAAA.

It has been tacitly assumed that the two different polypeptides, A and B, are under separate genetic control. This study presents genetic data which support this assumption. It also reinforces the concept of a tetrameric structure of the LDH molecule.

Materials and Methods.—The animals were obtained from a laboratory population of deer mice, *Peromyscus maniculatus*. All animals studied were at least 2 months old, and females were nonpregnant and nonlactating at the time of study.

The mice were killed by decapitation, and tissues to be analyzed were removed immediately, washed in cold 0.9% saline solution, blotted, weighed, and homogenized in a glass homogenizer with the appropriate amount of cold distilled water, which was usually 3–10 times the volume of the tissue. Liver, kidney, brain, and testis were routinely studied. The homogenate was placed in a polypropylene centrifuge tube, frozen and thawed twice, and centrifuged at 25,000 $\times$ *g* for ten min at 4–8°C. The clear supernatant was removed, and 40 microliters were added to the starch-gel insert. Vertical electrophoresis was carried out for 18 hr at a voltage

gradient of 10 volts per centimeter at 4°C. A continuous borate buffer system was used, and the gel buffer was 0.015 *M* at pH 8.4.

The gel was sliced horizontally into halves and incubated for 2 hr at 35°C in 100 ml of a solution containing 40% sodium DL-lactate 10 ml, DPN 25 mg, phenazine methosulfate 2 mg, nitro blue tetrazolium 25 mg, and 0.5 *M* tris, pH 7.1, 10 ml. Zones of LDH activity appeared as purple bands.

A screening study was carried out on individuals from a number of *Peromyscus* families kept in the laboratory, and a variant pattern of LDH, subsequently shown to be that of a heterozygote, was found in two related sibships. One of these belonged to the subspecies *P. maniculatus bairdi*, the other came from a cross between *P. m. bairdi* and a subspecific hybrid. Brother-sister matings of the offspring and backcrosses with both parents were carried out. Since it was necessary to sacrifice the animals before testing them for enzyme activity, the crosses had to be made "blind," hoping that, by chance, some of the mated animals would contain the variant pattern. Brain and kidney from each animal were analyzed, and animals of any one genotype invariably showed identical patterns except for minor quantitative differences.

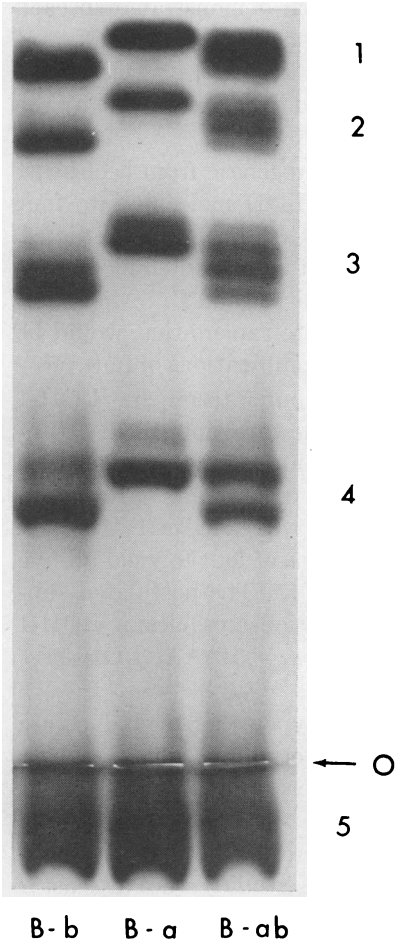

FIG. 1.—Zone electrophoresis patterns of *Peromyscus* brain extract on starch gel, showing three phenotypes of LDH isozymes: B-a normal, B-b homozygous variant, and B-ab heterozygous. Anode is at top, origin is at 0. Several extra or "ghost" bands are seen just anodal to some major bands, most prominent in this gel at Band 3. These occur inconstantly and are believed to represent *in vitro* artifacts.

Results.—Three patterns of LDH isozymes were observed (Fig. 1) involving variations in Bands 1, 2, 3, and 4, but not 5. In the normal pattern, here called B-a, and in the one considered to be the homozygous variant, B-b, the bands are similar in intensity and configuration, but the first four bands of this variant migrate more slowly than the corresponding bands in the normal mice. In the heterozygote, B-ab, Band 4 has 2 components of equal intensity, Band 3 is a triplet with the middle of the three being darker than the other two, Band 2 occurs as four components, the two middle ones being darkest, and Band 1 appears as a single, wide, diffuse zone which should theoretically contain five components. At each isozymic position the fastest component in the heterozygote lies parallel to the corresponding band in normal mice, while the slowest component advances at the same rate as the corresponding band of the homozygous variant.

Offspring have been obtained from three of the six possible mating types: B-a x B-a, B-a x B-ab, and B-ab x B-ab. The results of the matings are shown in Table 1. They demon-

TABLE 1

RESULTS OF GENETIC ANALYSIS

Mating type	Number of sib-ships	Number of offspring	Phenotypes of Offspring: Normal Male	Normal Female	Heterozygote Male	Heterozygote Female	Homozygous variant Male	Homozygous variant Female	Ratios of Phenotypes (normal:heterozygote:homozygous variant) Expected	Observed
Normal × normal	2	24	13	11	0	0	0	0	24:0:0	24:0:0
Normal × heterozygote	6	54	12	15	11	16	0	0	27:27:0	27:27:0
Heterozygote × heterozygote	2	12	2	1	0	5	2	2	3:6:3	3:5:4

strate that the variations in LDH pattern segregate in the expected Mendelian ratios. The six crosses between normals and heterozygotes included five matings involving heterozygous males. Among their progeny, which totaled 51, males and females were distributed approximately equally among the two expected classes. This demonstrates that the inheritance is autosomal.

Terminology.—The symbol *L-1* is suggested for the locus involved; *L* indicates lactate dehydrogenase, and the number one denotes the first locus involved in this group of enzymes discovered in the deer mouse. The normal gene at this locus is designated $L\text{-}1^a$, and its product is the B^a polypeptide. The variant allele is $L\text{-}1^b$, producing polypeptide B^b. This system retains the B notation for the polypeptide, as established by Markert.[3] Any subsequently discovered alleles would be designated by higher superscript letters. The genotypes, their polypeptide products, and phenotypes of the three LDH patterns would be as follows:

	Genotype	*Polypeptides produced*	*Phenotype*
Normal	$L\text{-}1^a/L\text{-}1^a$	B^a	B-a
Heterozygote	$L\text{-}1^a/L\text{-}1^b$	B^a, B^b	B-ab
Homozygous variant	$L\text{-}1^b/L\text{-}1^b$	B^b	B-b

Discussion.—The findings indicate that LDH Bands 1–4 in *Peromyscus* share a common polypeptide which is under the control of an autosomal locus, and that Band 5 does not contain this polypeptide. The variant thus involves an alteration of the B polypeptide.

In the heterozygote, the two allelic genes presumably produce equal amounts of their respective polypeptides, and the tetrameric molecules would be composed in part of "hybrid" forms produced by combinations of the normal and altered polypeptides, B^a and B^b. The theoretical structures of the various isozymes in the three types of patterns, with the expected binomial proportions of each, assuming random combination, are shown in Table 2.

It can be seen in Figure 1 that Bands 2–5 fit the above scheme. Band 1 appears in the heterozygote as a single diffuse zone, and is presumed to contain five bands which were not resolvable in the electrophoretic system used in this investigation. The findings thus support the hypothesis of a tetrameric structure of the LDH isozymes, the molecules being composed of various combinations of two distinct polypeptides (A and B) which are under separate genetic control.

Whether the variant LDH molecules confer any selective advantage or disadvantage is not evident. The enzyme activity of the altered forms is apparently unchanged, and the homozygous animals, both normal and variant, appeared healthy.

TABLE 2

PROPOSED TETRAMERIC STRUCTURES OF THE THREE PATTERNS OF LDH ISOZYMES SHOWING RELATIVE POSITIONS OF SUB-BANDS WITHIN EACH MAJOR BAND

	Normal (genotype *L-1a/L-1a*)	Heterozygote* (genotype *L-1a/L-1b*)	Homozygous Variant (genotype *L-1b/L-1b*)
LDH-1	$B^a\ B^a\ B^a\ B^a$	$^1/_{16}$ $B^a\ B^a\ B^a\ B^a$ $^4/_{16}$ $B^a\ B^a\ B^a\ B^b$ $^6/_{16}$ $B^a\ B^a\ B^b\ B^b$ $^4/_{16}$ $B^a\ B^b\ B^b\ B^b$ $^1/_{16}$ $B^b\ B^b\ B^b\ B^b$	$B^b\ B^b\ B^b\ B^b$
LDH-2	$A\ B^a\ B^a\ B^a$	$^1/_8$ $A\ B^a\ B^a\ B^a$ $^3/_8$ $A\ B^a\ B^a\ B^b$ $^3/_8$ $A\ B^a\ B^b\ B^b$ $^1/_8$ $A\ B^b\ B^b\ B^b$	$A\ B^b\ B^b\ B^b$
LDH-3	$A\ A\ B^a\ B^a$	$^1/_4$ $A\ A\ B^a\ B^a$ $^2/_4$ $A\ A\ B^a\ B^b$ $^1/_4$ $A\ A\ B^b\ B^b$	$A\ A\ B^b\ B^b$
LDH-4	$A\ A\ A\ B^a$	$^1/_2$ $A\ A\ A\ B^a$ $^1/_2$ $A\ A\ A\ B^b$	$A\ A\ A\ B^b$
LDH-5	$A\ A\ A\ A$	$A\ A\ A\ A$	$A\ A\ A\ A$

* Subunit polypeptides are A, B^a, and B^b. Ratios preceding the heterozygous tetramers indicate relative proportions of each sub-band within that major band.

Summary.—A genetically-determined variant pattern of LDH isozymes was found in kidney and brain extracts of certain stocks of the deer mouse, *Peromyscus maniculatus*. Analysis by starch-gel electrophoresis shows that four of the five isozymic bands are involved, the altered molecules migrating more slowly than the normal ones. Heterozygous individuals contain the normal and variant bands, as well as additional ones formed by the combination of normal and altered polypeptides in the same molecule.

The frequency distribution of the various LDH patterns among offspring from different types of matings demonstrates that the observed variations are genetically determined, involving a single autosomal locus which controls the polypeptide found in the four most negatively-charged isozymes. In addition, the findings conform with the hypothesis that the LDH isozymes are tetramers composed of the five possible combinations of two different subunits.

Note added in proof: LDH-1 in the heterozygote has now been resolved into five bands, in approximately the expected proportions of 1:4:6:4:1. This was done by increasing the gel buffer strength to 0.1 *M*. Also, by using a phosphate buffer system (0.01 *M*, pH 8.5), LDH-5 has been caused to migrate anodally. It occurs as a single, discrete band, and clearly shows no genetic variation in this study.

The authors wish to acknowledge the helpful suggestions of Prof. Clement L. Markert of the Johns Hopkins University, and the excellent technical assistance of Mrs. Lucille Setter.

* This investigation was supported in part by a grant from the Scottish Rite Foundation for Research in Schizophrenia, and by USPHS research grant NB-03095, National Institutes of Health.

[1] Markert, C. L., and F. Møller, these PROCEEDINGS, **45**, 753–763 (1959).

[2] Appella, E., and C. L. Markert, *Biochem. Biophys. Res. Comm.* **6**, 171–176 (1961).

[3] Markert, C. L., in *Hereditary, Developmental and Immunologic Aspects of Kidney Disease*, ed. J. Metcoff (Evanston, Illinois: Northwestern University Press, 1962), pp. 54–63.

[4] Cahn, R. D., N. O. Kaplan, L. Levine, and E. Zwilling, *Science*, **136**, 962–969 (1962).

[5] Markert, C. L., *Science*, **140**, 1329–1330 (1963).