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Hereditary Lactate Dehydrogenase A-Subunit Deficiency as Cause of Early Postimplantation Death of Homozygotes in *Mus musculus*

Siegbert Merkle,* Jack Favor,* Jochen Graw,* Sabine Hornhardt† and Walter Pretsch*,[1]

**Institut für Säugetiergenetik and †Abteilung für Zellchemie, GSF-Forschungszentrum für Umwelt und Gesundheit GmbH, D-8042 Neuherberg, Germany*

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ABSTRACT

Two ethylnitrosourea-induced heterozygous mouse mutants with approximately 58 and 50% of wild-type lactate dehydrogenase (LDH) activity and a γ-ray-induced heterozygous mutant with 50% of wild-type LDH activity in blood, liver and spleen (expressing predominantly the *Ldh-1* gene) were recovered in mutagenicity experiments following spermatogonial treatment. Physiological and genetic studies revealed no indications for differences in fertility as well as hematological or other physiological traits between heterozygotes of each mutant line and wild types. This suggests that neither the mutations in the heterozygous state *per se* nor the resulting approximate 42 to 50% LDH deficiency affect metabolism and fitness. Physicochemical and immunological studies clearly demonstrated that the two mutations with 50% deficiency in heterozygotes result from null alleles of the *Ldh-1* structural locus, generating neither enzyme activity nor immunological cross-reacting material. In contrast, the heterozygous mutant with approximately 58% of normal blood LDH activity was shown to be due to a *Ldh-1* allele creating protein subunits, which in random assortment with wild-type subunits *in vivo* exhibit a reduced specific activity and further alterations of kinetic and physicochemical characteristics. All the mutations in the homozygous state were found to be lethal at an early postimplantation stage of embryonic development, probably due to a block of glycolysis with the corresponding loss of the main source of metabolic energy during this ontogenetic stage. The distinct physiological consequences of the total absence of a functioning LDH-A subunit in mice and humans are discussed. The key role regarding the presence as well as developmental pattern of isozymes in estimating the impact of enzyme-activity mutations on the phenotype of an organism is emphasized.

LACTATE dehydrogenase (LDH, L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27.) catalyzes the reversible conversion of lactate to pyruvate and thereby plays an important role both in anaerobic glycolysis as well as in lactate oxidation. In mammals there are three separate structural LDH genes, the expression of which is developmentally regulated and tissue specific (LI 1989).

Several electrophoretic LDH variants, as well as cases of complete deficiency of either the A or B subunit have been reported in humans (VESELL 1965; KITAMURA *et al.* 1971; TANIS, NEEL and TORRES DE ARAUZ 1977; KANNO *et al.* 1980; MOHRENWEISER and NOVOTNY 1982; MAEKAWA, SUDO and KANNO 1986) and other mammals (SHAW and BARTO 1963; RAUCH 1968; CATTANACH and PEREZ 1969; BRITTON-DAVIDIAN *et al.* 1978), including the house mouse *Mus musculus* (SOARES 1977, 1978; BRITTON-DAVIDIAN *et al.* 1978; PRETSCH and CHARLES 1980; PRETSCH 1989). However, a detailed characterization of the LDH-deficient mutants of the mouse and a comparison with humans suffering from LDH deficiency is still lacking. The comparison of the significance of LDH deficiency with respect to metabolism and physiology in mouse and man might be particularly important since information on the impact of induced or spontaneous mutations at homologous genetical endpoints would improve understanding of the genetic risks of radiation and chemicals for humans.

In the present study results of the genetic, physiological and biochemical characterizations of three LDH-deficient mouse mutants are reported. Regardless of the nature of the individual mutations, all were found to be homozygous lethal at an early postimplantation stage of embryonic development. This phenomenon might be due to a block in glycolysis and the associated loss of the main source of metabolic energy because of a total or almost total loss of LDH mediated $NADH^+$ reoxidation.

MATERIALS AND METHODS

Animals: Two mouse mutants (LDH 1049 and LDH 9546) with approximately 58 and 50% wild-type LDH activity in blood were recovered independently in mutagenesis experiments, in which offspring from ethylnitrosourea (ENU)-treated (102/El × C3H/El)F_1 hybrid males and untreated test stock females were screened for enzyme activity variants (CHARLES and PRETSCH 1987). A third independent

[1] Author to whom reprint requests should be sent.

mutant (LDH 2534) with 50% of normal blood LDH activity was a progeny of an AKR male irradiated with a split-dose of 3 + 3 Gy γ-rays with a 24-hr fractionation interval and an unexposed test stock female (unpublished). For biochemical and physiological characterization 10-week-old animals of both sexes were used, which were backcrossed at least 10 generations to the C3H/El wild-type strain (F_{10}–F_{15}). After weaning, four mice of the same sex were housed per cage and maintained under constant temperature (22 ± 2°) with a fixed 12-hr light/12-hr dark cycle. Tap water and standardized diet (Altromin 1314, Altromin International, Lage, Germany) were provided *ad libitum*. Based on the conclusions drawn from the results of the present and previous investigation (PRETSCH 1989) the mutant alleles of the ENU-induced mutant lines LDH 1049 and LDH 9546 (CHARLES and PRETSCH 1987) and the radiation-induced mutant line LDH 2534 (unpublished) were designated *Ldh-1*$^{a\text{-}m2Neu}$, *Ldh-1*$^{a\text{-}m3Neu}$ and *Ldh-1*$^{a\text{-}m4Neu}$, respectively. The mutations will be referred to as LDH-A2N, LDH-A3N and LDH-A4N, respectively.

Genetic and physiological analysis: Genetic and physiological characterization of the mutants including determination of the stage of homozygous lethality, sampling and preparation of blood, erythrocytes and tissues, as well as the studies of hematologic and other physiological parameters followed essentially the procedures previously described in detail (EHLING *et al.* 1978; MERKLE and PRETSCH 1989).

Physicochemical and kinetical properties: Enzymatic activity was determined using the automatic analyzer ACP 5040 (Eppendorf, Hamburg, Germany). The standard reaction mixture for measuring LDH activity contained IMI buffer (0.1 M imidazol, pH 7.0, 70 mM KCl, 18 mM $MgSO_4$), 8.5 mM pyruvate (Na salt) and 0.28 mM NADH (Na_2 salt). Measurement of the Michaelis-Menten constants (K_m) for pyruvate and NADH were performed using the standard assay for LDH with varying concentrations of pyruvate (0.05; 0.1; 0.2; 0.4; 2 mM) or NADH (0.007; 0.014; 0.028; 0.042; 0.28 mM), respectively. The apparent K_m was estimated by a least-squares regression fit.

The substrate specifity of LDH was tested by comparing the activity of the enzyme with 0.7 mM glyoxalate as substrate with that obtained in an assay containing 0.3 mM pyruvate as substrate (MOHRENWEISER and NOVOTNY 1982).

Noncompetitive inhibition of LDH by oxalate (NISSELBAUM, PACKER and BODANSKY 1964) was studied in the standard assay containing 0.1 mM pyruvate and 0.7 mM oxalate (Na salt). The inhibition of LDH by high concentrations of pyruvate was measured in the standard assay and is given as percent activity with 8.5 or 20 mM pyruvate as substrate compared to the maximal activity with 2 mM pyruvate as substrate. All determinations of kinetic and inhibitory properties were performed in erythrocyte lysates at 25°. LDH activity in the assays of homozygous wild types were adjusted to that of heterozygous mutants by diluting the lysates.

Heat stability of erythrocyte LDH was determined by incubating erythrocyte lysate at 60 and 62°. The concentration of hemoglobin in the lysates was approximately 0.015 g/ml. At 5-min time intervals aliquots were taken and chilled immediately with ice-cold 0.1 M IMI buffer. After sedimentation of precipitated hemoglobin, residual LDH activity was assayed as described above.

A Tris-glycine-phosphate buffer system (BEUTLER, MATHAI and SMITH 1968) with substrate concentrations of the standard assay was used to determine the pH dependence of LDH.

Isoelectric focusing was carried out on ultrathin-layer polyacrylamide gels with a final pH range of 3.0–9.0 according to the procedure of RADOLA (1980) using Servalyt 4–9T (Serva, Heidelberg, Germany). Polyacrylamide gel electrophoresis (PAGE) and staining of the gels were performed as described by CHARLES and PRETSCH (1981).

Immunological properties: Antiserum against commercial, cytoplasmic pig muscle LDH (Boehringer, Mannheim, Germany) was raised in rabbits. An aliquot containing approximately 1 mg of protein was mixed with the same volume of Freund's adjuvant and injected subcutaneously. The procedure was repeated at 2-week intervals for a total of five injections. Blood samples were drawn after the final injection via venipuncture. After centrifugation the antiserum was stored at −80°. To determine the amount of LDH-cross-reacting material, Western blot analysis was performed as described by TOWBIN, STAEHELIN and GORDON (1979) using a Sartoblot semidry blot apparatus (Sartorius, Göttingen, Germany) after sodium dodecyl sulfate (SDS)-PAGE according to LAEMMLI (1970) with 200 μg spleen protein. Protein concentration of the samples was determined according to LOWRY *et al.* (1951). The antibodies bound to the nitrocellulose sheet were stained according to the method of STERNBERGER, CUCULIS and MEYER (1970) using peroxidase conjugated antisera (Bio-Makor, Rehovot, Israel). The stained bands were scanned at 546 nm using Elscript 400 densitometer (Hirschmann, Unterhaching, Germany) and the peaks were integrated.

Statistical analysis: In the biochemical and physiological characterization experiments, data from the same number of female and male animals were used for the mean and SEM. For statistical comparisons between the different genotypes, Student's *t*-test was used. Data from the homozygous-lethality experiments were compared using a nonparametric method (Wilcoxon-rank-sum-test). Differences were stated as significant for P values less than 0.05.

RESULTS

Genetic characterization and stage of homozygous lethality *in utero*: Table 1 summarizes the results of the genetic studies. Backcrossing heterozygous animals from the three independent *Ldh-1* mutant lines with wild-type C3H/El animals yielded normal litter sizes with homozygous wild-type and heterozygous mutant offspring in a ratio of approximately 1:1. In contrast, intercrossing heterozygotes of each mutant line resulted in a reduction of litter size. Only wild types and heterozygotes in an approximate 1:2 ratio were obtained in the mutant lines LDH-A2N and LDH-A4N. In the LDH-A3N mutant line, however, beside 236 mice with normal and 469 mice with half of normal LDH activity 4 animals with approximately 1% residual LDH activity were recovered. Since the latter died within a few weeks after birth, it was impossible to genetically determine their genotype. The profound reduction of LDH activity and the concomitant decrease of hemoglobin concentration in blood (9.9 ± 0.5 g Hb/100 ml *vs.* 15.3 ± 0.6 g Hb/100 ml of wild types and heterozygotes) and the visible reduction of body size suggest that these animals were homozygous mutants. In this context it must be noted that three out of these 4 animals occurred in intercrosses of F_1 heterozygotes and the fourth case in intercrosses of F_5 animals. Among approximately 300

TABLE 1

Genetic characterization of three LDH-A deficiency mutants in mice

Cross *Ldh-1* genotype[a]	Mean litter size ± SD	+/+ (*n*)	+/− (*n*)	−/− (*n*)	Ratio +/+:+/−	Reduction of litter size (%)[b]
+/+ × +/+	6.5 ± 1.9	130				
LDH-A2N						
+/− × +/+	6.5 ± 1.9	419	455		1:1.09	
+/− × +/−	5.3 ± 1.4	108	191	0	1:1.77	19
LDH-A3N						
+/− × +/+	6.8 ± 1.7	308	312		1:1.01	
+/− × +/−	5.4 ± 2.0	236	469	4	1:1.99	21
LDH-A4N						
+/− × +/+	6.8 ± 1.9	136	132		1:0.97	
+/− × +/−	5.2 ± 2.3	48	104	0	1:2.17	24

[a] +/+ = wild type; +/− = heterozygous mutant; −/− = homozygous mutant.
[b] Reduction of litter size of intercrosses compared to backcrosses is calculated as follows: (1 − litter size of intercrosses:litter size of backcrosses) × 100.

offspring of intercrosses from later generations (F_6–F_{15}), so far no additional animals with this phenotype could be identified.

The reduction in litter size together with the absence or the significantly reduced number of animals with a third phenotype in intercrosses suggest that mutant homozygotes are lethal. Indeed, the genetic testing of 20 randomly chosen offspring from intercrosses with about half of normal LDH activity indicated heterozygosity for these animals. Therefore, the possibility can be excluded that the mutations are dominantly expressed and that homozygous mutants have the same phenotype as heterozygotes (FAVOR 1984). The time of death of homozygous mutant embryos was determined by examining uterine contents and corpora lutea at 14 days *post conceptionem*. All three mutant lines revealed no significant differences in the number of corpora lutea, preimplantation loss or late death implants between control and intercrossed dams (Table 2). In contrast, the number of live implants was significantly reduced by approximately 25% and the number of early postimplantation deaths significantly increased in dams of intercrosses. The mean absolute increase in early death implants is approximately as high as the mean absolute decrease in live implants, suggesting that lethality of homozygous *Ldh-1* mutants generally occurs at an early postimplantation stage of embryonic development.

Physiological characterization: To determine a possible effect of LDH-A subunit deficiency on erythrocyte metabolism or conversely to exclude the possibility that the reduced LDH activity in blood is modulated by an altered erythrocyte population, routine hematological tests were performed. In heterozygous mutants no deviations from the wild-type values were observed for the hematocrit (wild-type value [WTV] = 47.5 ± 0.8%), hemoglobin (WTV = 15.2 ± 0.3 g/liter), number of red blood cells (WTV = 8.4 ± 0.2 × 10^{12} cells/liter), mean cellular fragility (WTV = 0.497 ± 0.007% NaCl) and glucose consumption of blood (WTV = 2.9 ± 0.4 mg/g Hb/hr). Other physiological traits such as plasma glucose (WTV = 1.35 ± 0.18 g/liter), body weight (WTV = 22.8 ± 0.6 g) and organo-somatic indices (organ weight × 100/body weight) of liver (WTV = 5.65 ± 0.20 g/100 g body weight), lung (WTV = 0.58 ± 0.02 g/100 g body weight), kidney (WTV = 1.36 ± 0.04 g/100 g body weight), spleen (WTV = 0.40 ± 0.02 g/100 g body weight) and heart (WTV = 0.39 ± 0.01 g/100 g body weight) also indicated no difference between wild types and mutants. Furthermore, the concentration of extractable proteins given in g/100 g wet weight in liver (WTV = 17.1 ± 0.8), lung (WTV = 11 .6 ± 0.4), kidney (WTV = 15.3 ± 0.5), spleen (WTV = 13.1 ± 0.5), heart (WTV = 10.9 ± 0.4), muscle (WTV = 7.8 ± 0.3) and brain (WTV = 6.9± 0.3) were not significantly altered in heterozygotes when compared to the wild type. These findings suggest that neither the mutations in the heterozygous state *per se* nor the resulting 42–50% LDH-A deficiency affect physiological functions. In turn, the results obtained in the hematological tests indicate that the reduction of LDH activity is not an indirect result of an altered red blood cell population.

LDH activities in blood and other tissues: The levels of LDH activity in blood, erythrocytes, plasma and several tissues of wild types and heterozygous mutants are shown in Table 3. A comparable LDH reduction of approximately 42 or 50% as observed in blood and erythrocytes was also found in liver, spleen and muscle of heterozygous animals. On the contrary, brain, heart and kidney exhibited smaller reductions in LDH activity ranging from 15 to 30% depending on tissue and mutant line, whereas the values of lung

TABLE 2

***In utero* lethality of homozygotes for three LDH-A deficiency mutations in mice**

Cross *Ldh-1* genotype[a]	No. of females	Corpora lutea	Preimplantation loss[b]	Dead implants		Live implants
				Early	Late	
LDH-A2N						
+/+ × +/+	11	9.0 ± 0.3	0.4 ± 0.3	0.6 ± 0.2	0.2 ± 0.1	7.8 ± 0.5
+/− × +/−	11	8.8 ± 0.4	0.1 ± 0.1	2.9 ± 0.4*	0.2 ± 0.1	5.6 ± 0.3*
LDH-A3N						
+/+ × +/+	10	11.1 ± 0.6	1.1 ± 0.3	0.9 ± 0.4	0	9.1 ± 0.7
+/− × +/−	10	10.7 ± 0.8	0.9 ± 0.4	3.0 ± 0.6*	0	6.8 ± 0.5*
LDH-A4N						
+/+ × +/+	8	9.1 ± 0.4	0.6 ± 0.4	0.1 ± 0.1	0.3 ± 0.2	8.1 ± 0.6
+/− × +/−	8	9.6 ± 0.5	0.3 ± 0.2	3.4 ± 0.4*	0	5.9 ± 0.4*

Data are given per female as mean ± SEM. Significant differences ($P \leq 0.05$) between females originating from control crosses and intercrosses are marked by *.

[a] Same symbols are used as in Table 1. For each mutant line, control animals and heterozygous mutants originated from the same litter.

[b] Preimplantation loss was determined using the difference between number of corpora lutea and number of total implants.

TABLE 3

LDH activity in blood, erythrocytes, plasma and several tissues from wild types and heterozygotes for LDH-A deficiency mutations in mice

Tissue	*Ldh-1* genotype[a]			
	+/+	LDH-A2N +/−	LDH-A3N +/−	LDH-A4N +/−
Blood	100.0 ± 3.0 (226)	58.2 ± 1.9	50.5 ± 1.2	49.5 ± 2.0
Erythrocyte	100.0 ± 4.6 (206)	57.6 ± 2.5	49.6 ± 2.1	49.0 ± 2.4
Plasma	100.0 ± 9.5 (0.82)	64.4 ± 8.2	56.8 ± 6.8	57.8 ± 9.7
Liver	100.0 ± 5.3 (1451)	58.0 ± 4.1	52.0 ± 3.8	49.8 ± 2.4
Lung	100.0 ± 5.9 (642)	67.4 ± 3.2	58.7 ± 3.5	56.3 ± 2.9
Kidney	100.0 ± 7.6 (1683)	82.9 ± 3.6	73.3 ± 3.5	74.7 ± 2.4
Spleen	100.0 ± 2.9 (789)	57.0 ± 3.1	51.9 ± 3.6	52.1 ± 2.7
Heart	100.0 ± 4.6 (2228)	84.6 ± 3.3	79.4 ± 1.7	77.7 ± 2.2
Muscle	100.0 ± 3.6 (6796)	61.5 ± 3.1	57.6 ± 3.0	58.0 ± 2.5
Brain	100.0 ± 4.9 (1353)	78.8 ± 4.8	72.0 ± 2.2	71.1 ± 2.7

Data are expressed as percentage of LDH wild-type activity and given as mean ± SEM of 10 animals. In parenthesis the mean specific activity is given as units/g Hb in blood and erythrocytes, units/ml in plasma and in units/g protein in other tissues. No significant differences due to genotype were found in the amount of protein extracted per gram tissue.

[a] Same symbols are used as in Table 1.

and plasma were intermediate between both extremes. The differential tissue-specific decrease of LDH activity in the mutants thus reflects the well known relative expression of the two LDH isozymes in murine tissues (MARKERT and URSPRUNG 1962). Organs or cells in which mainly or exclusively the A subunit occurs show the largest decrease of activity, whereas the predominance of the B subunit correlates with a minor deficiency in the respective tissue. Moreover, a striking consistency was found between heterozygous animals of the ENU-induced LDH-A3N and the radiation-induced LDH-A4N mutants. Mice of both mutant lines exhibited essentially the same reduction of LDH activity in all tissues studied. The approximate 50% LDH reduction in red blood cells, liver, spleen and muscle corroborates the exclusive or nearly exclusive expression of the LDH-A isozyme in these tissues and suggests that the underlying mutations generated null alleles at the *Ldh-1* locus in both mutant lines. However, the occurence of a null allele can be excluded for the LDH-A2N mutant which expressed approximately 58% activity in tissues containing nearly exclusively the A subunit, such as blood, liver and spleen.

Physicochemical and kinetical properties of LDH: The physicochemical and kinetic characteristics of erythrocyte LDH of A-3N and A-4N mutants including K_m for pyruvate (WTV = 0.200 ± 0.002 mM), K_m for NADH (WTV = 36.6 ± 2.0 μM), inhibition by high concentrations of pyruvate (8.5 mM: WTV = 74.3 ± 2.0%; 20 mM: WTV = 52.0 ± 1.2%), substrate specifity (glyoxalate/pyruvate: WTV = 3.4 ± 0.2%), noncompetitive inhibition by oxalate (WTV = 92.5 ± 0.7%), heat stability at 60.0° and 62.0°, pH dependence, electrophoretic mobility and isoelectric focusing pattern (Figure 1) were not significantly different from those of the wild type supporting the hypothesis that these mutants are true nulls. In contrast the A-2N mutant exhibits slight but significant differences in some of the enzyme properties studied, *i.e.*, isoelectric focusing pattern (Figure 1), heat stability (Figure 2), pH dependence (Figure 3) and inhibition by high concentrations of pyruvate (8.5 mM: mutant value

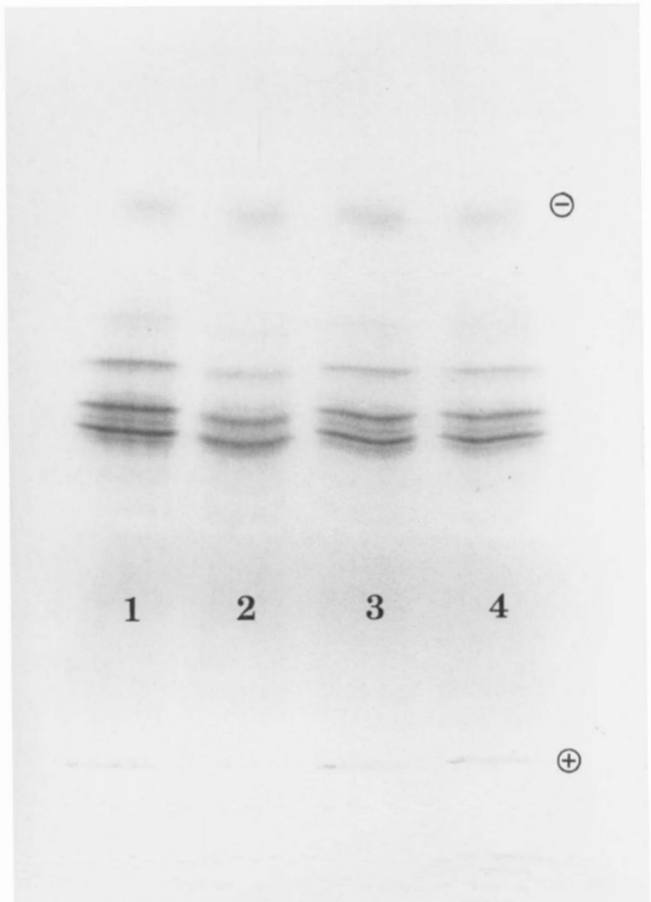

FIGURE 1.—Isoelectric focusing pattern of erythrocyte LDH of wild types and heterozygotes for three *Ldh-1* mutants. Lane 1, *Ldh-1*^{a}/*Ldh-1*^{a}; lane 2, *Ldh-1*^{a}/*Ldh-1*$^{a\text{-}m2Neu}$; lane 3, *Ldh-1*^{a}/*Ldh-1*$^{a\text{-}m3Neu}$; lane 4, *Ldh-1*^{a}/*Ldh-1*$^{a\text{-}m4Neu}$.

[MV] = 83.7 ± 0.9%; 20 mM: MV = 64.0 ± 1.2%).

Immunological properties: To confirm the hypothesis that LDH-A3N and LDH-A4N are null mutations and that LDH-A2N is different from the other two, Western blot analysis was conducted using spleen tissue since it exhibits the same LDH isozyme distribution pattern as in blood and erythrocytes (MARKERT and URSPRUNG 1962). As seen in Figure 4, Western blots reveal similar molecular sizes of the detectable LDH enzymes and no additional band as compared to the wild type. Further, the quantitative analysis (Table 4) clearly demonstrates that the amount of detectable LDH is similar in wild types and LDH-A2N mutants, whereas it is about half in LDH-A3N and LDH-A4N animals. The comparison between LDH activity and the amount of LDH-cross-reacting material in LDH-A3N and LDH-A4N individuals suggests that the decrease of LDH activity in these two mutants represents a reduction in immunologically detectable protein. Moreover, it indicates that the decrease of LDH activity in LDH-A2N mutants is not a result of a reduced concentration of LDH molecules, but rather is due to diminished specific enzyme activity of mutant LDH molecules.

DISCUSSION

Site and nature of the mutations: The detection of a mutant organism exhibiting reduced activity of an enzyme which occurs in more than one isozymic form always implicates the question as to which isozyme is affected by the mutation. In the case of hereditary LDH deficiency in the murine blood, however, the answer is evident *a priori*. Because of the predominant occurrence of the LDH-A isozyme in mouse erythrocytes (ENGEL, KREUTZ and WOLF 1972), a detectable LDH deficiency in blood of this species can be attributed to a deficiency of A subunits caused by a mutation affecting the *Ldh-1* structural locus. In the case of the two ENU-induced mouse mutants LDH-A2N and LDH-A3N this has been confirmed by linkage analysis (PRETSCH 1989); the genetic confirmation was not performed in the radiation-induced LDH-A4N mutant line but the extensive biochemical analyses presented here confirm this hypothesis.

Regarding the mutants with approximately 50% of normal LDH activity, LDH-A3N and LDH-A4N, the study of physicochemical, kinetic and immunological properties as well as the results of the enzyme activity measurements in a variety of tissues suggest mutations generating null alleles with neither detectable enzyme activity nor immunologically cross-reacting material. However, since null alleles may result from a broad spectrum of DNA alterations including nonsense mutations, frame shifts or deletions, these findings may only give limited insight into the nature of the mutations. Yet, some deductions as to the latter might be made from the origin of the mutations. Determination of the exact nature of ENU-induced mutations by DNA-sequence analysis showed that the predominant mutations were base pair exchanges (GOSSEN *et al.* 1989; PASTINK *et al.* 1989; SIMONS *et al.* 1991). In contrast, the mutation spectrum following exposure to X-rays is more complex. As summarized by GROSOVSKY *et al.* (1988) several reports estimate the percentage of alterations detectable by Southern blot analysis (>50 bp) between 16 and 80% in ionizing radiation-induced mutations of mammalian cells. Among the large number of radiation-induced mutations alterations of which are not detectable by Southern blot analysis (<50 bp), the majority (about 2/3) represents base substitutions.

Physiological effects of LDH-A subunit deficiency: Even if the defined nature of the mutations encountered in this study could not be elucidated in detail there seems to be yet sufficient evidence that at least two of the three LDH mutants are the result of mutations affecting exclusively the *Ldh-1* structural gene. Consequently, the resultant physiological phe-

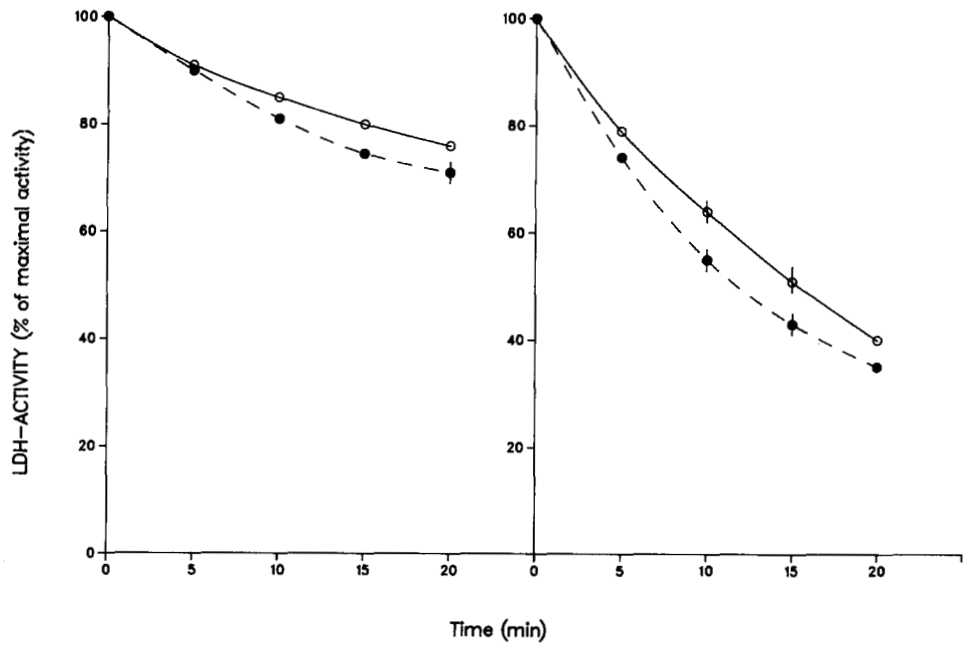

FIGURE 2.—Percent residual activity of erythrocyte LDH of wild types (open circles) and heterozygous *Ldh-1*$^{a\text{-}m2Neu}$ mutants (closed circles) after incubation at 60° (left) and 62° (right). Values are given as means of 6 animals (double determination). Bars represent ±1 SEM where visible, or are smaller than the plotted symbol.

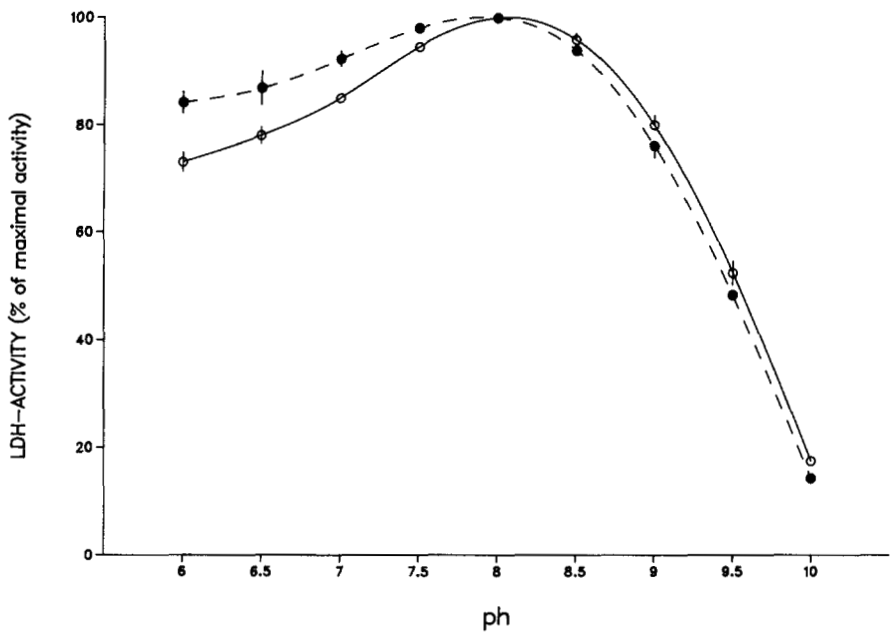

FIGURE 3.—Percent of maximal activity of erythrocyte LDH of wild types (open circles) and heterozygous *Ldh-1*$^{a\text{-}m2Neu}$ mutants (closed circles) at different pH values. Values are given as means of six animals (double determination). Bars represent ±1 SEM where visible, or are smaller than the plotted symbol.

notype of the mice carrying the respective mutations should be attributable to a clear cause-effect relationship between enzyme deficiency and metabolic function.

The presence or severity of a metabolic dysfunction depends on the degree of the enzyme deficiency. The study of enzyme deficiency and hereditary disease in humans presented evidence that at least for enzymes other than those which are rate-limiting, more than 50% reduction of activity is necessary to produce metabolic symptoms (STANBURY, WYNGAARDEN and FREDRICKSON 1972). Not surprisingly, the 42–50% reduction of LDH-A activity in heterozygous *Ldh-1* mutants given in this paper produce no obvious harm to the carriers. In contrast, the expected total loss of LDH-A activity in homozygous LDH-A3N or LDH-A4N animals, as well as the expected severe LDH-A deficiency in LDH-A2N homozygotes was shown to have lethal consequences for these animals as early postimplantation embryos.

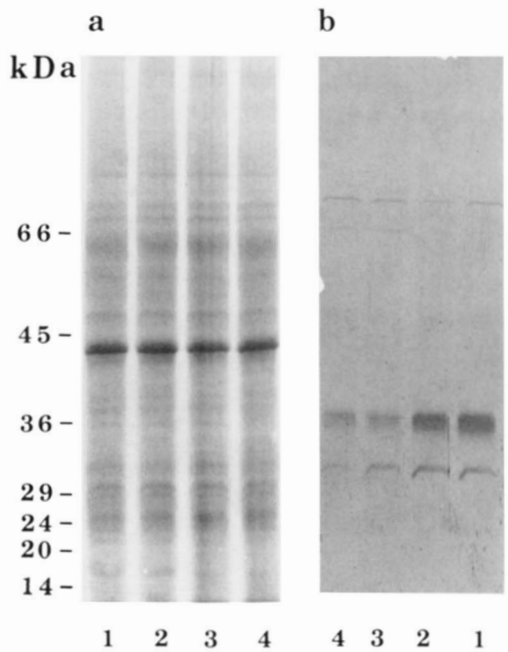

FIGURE 4.—Western blot analysis of splenic LDH of wild types and heterozygotes for three *Ldh-1* mutants. SDS-PAGE of spleen proteins. a) Staining with Coomassie brilliant blue. Molecular mass markers are: bovine albumin (66 kD); egg albumin (45 kD); glyceraldehyde-3-phosphate dehydrogenase (36 kD); carbonic anhydrase (29 kD); trypsinogen (24 kD); trypsin inhibitor (20 kD); α-lactalbumin (14 kD). b) Analysis with LDH-specific antibodies. The LDH-specific band appears at 36 kD (molecular mass of LDH subunits). Lane 1, *Ldh-1*^{a}/*Ldh-1*^{a}; lane 2, *Ldh-1*^{a}/*Ldh-1*$^{a\text{-}m2Neu}$; lane 3, *Ldh-1*^{a}/*Ldh-1*$^{a\text{-}m3Neu}$; lane 4, *Ldh-1*^{a}/*Ldh-1*$^{a\text{-}m4Neu}$.

The prediction whether and at which stage of ontogenic development a mutational event generating a severe deficiency or total absence of a given enzymatic gene product may have deleterious consequences for metabolism and may result in lethality of the organism, is predicted upon the consideration of several points: (1) the role of the enzyme and of the corresponding metabolic pathway within the total metabolism of an organism, (2) the possibility of alternative pathways or of bypasses of the respective enzymatic step in the affected pathway, (3) the presence of isozymes as well as their tissue-specific and developmental patterns and (4) the ontogenetic transition from oocyte-coded to embryo-coded enzyme in the developing embryo.

The roles of LDH within the mammalian metabolism are well understood (VESSEL 1975; EVERSE and KAPLAN 1975). One of the most important is the regeneration of NAD^+ required for glycolysis to continue under anaerobic conditions. Since under these conditions the glycolytic pathway is the major source of cellular ATP and moreover the LDH step, with few exceptions, may not be compensated, deleterious consequences due to LDH deficiency are expected under environmental or functional anaerobiosis.

Although there have been no measurements of oxygen availability in the early postimplantation mouse embryos it may be assumed that until the formation of the chorioallantoic placenta, the embryonic tissue is almost certainly developing under anaerobic conditions (ELLINGTON 1987). Accordingly, previous metabolic studies suggested that the early postimplantation mouse embryo unlike the preimplantation mouse embryo depends upon glycolysis for survival (CLOUGH and WHITTINGHAM 1983; GARDNER and LEESE 1988). This suggestion was supported by the finding that mice homozygous for both *Tpi-1* and *Gpi-1s* null alleles die at an early postimplantation stage of embryonic development (MERKLE and PRETSCH 1989; WEST *et al.* 1990).

In comparison with TPI and GPI, which only occur in one isozymic form, the situation for LDH is more complex because of the presence of two isozymic forms in most tissues. Because the deficiency of the one enzyme due to a mutation could be easily compensated by the persistent presence of the second, the tissue-specific and/or the developmental pattern of the isozymes must be taken into consideration. Moreover, one might argue that LDH appears to play an important role in the early preimplantation embryo. During the two to eight cell stage when the embryo passes through the lactate-rich environment of the Fallopian tube, pyruvate appears to provide the major energy source for the mouse and other mammalian embryos (BRINSTER 1965, 1970; LEESE and BARTON

TABLE 4

Intensity of LDH-specific bands after Western blot analysis and the specific LDH activity in the spleen of heterozygotes for three LDH-A deficiency mutations in mice

Activity	*Ldh-1* genotype[a]			
	+/+	LDH-A2N +/−	LDH-A3N +/−	LDH-A4N +/−
Intensity of LDH-specific band (% of wild type)	100.0 ± 13.3	100.6 ± 5.9	52.9 ± 3.7*	59.4 ± 7.0*
LDH activity (U/g protein)	840.4 ± 60.6	485.8 ± 58.0*	467.3 ± 35.3*	486.6 ± 95.8*
Specific LDH activity (% of wild type)[b]	100.0 ± 10.5	57.5 ± 3.1*	105.9 ± 8.6	97.5 ± 12.1

Data are given as mean ± SEM of four animals. Significant differences between wild types and mutants are marked by *.

[a] Same symbols are used as in Table 1.

[b] Specific LDH activity was calculated by relating the LDH activity of spleen given in units/g protein to the intensity of LDH-specific band.

1984). Oxidation of exogenous lactate by embryonic LDH could be an important source of pyruvate. An effect of LDH-A deficiency on ontogenetic development of the preimplantation mouse, however, is not to be expected since LDH activity during this phase is contributed from oocyte-coded LDH-B subunits (for a review, see BIGGERS and STERN 1973; BRINSTER 1973, 1979). This predominance of the B subunit is lost during implantation when an increase in the synthesis of presumably embryo-coded A subunits results in a shift in the isozyme pattern such that 90% of the LDH activity is contributed by the A subunit (AUERBACH and BRINSTER 1967; RAPOLA and KOSKIMIES 1967; BRINSTER 1970; MONK and ANSELL 1976). As development proceeds, the contribution of B subunits to the pattern increases (MARKERT and URSPRUNG 1962; ENGEL and PETZOLD 1973; MONK and ANSELL 1976). Summarizing the available data, it is expected that a total absence or marked functional impairment of LDH-A in the mouse leads to a severe deficiency of total LDH activity during the early postimplantation stage of development.

The observed homozygus lethality due to *Ldh-1* alleles coding for no or dysfunctional LDH-A molecules is consistent with the predominance of the LDH-A isozyme and the dependence of the murine embryo on energy metabolism via glycolysis during the early postimplantation stage. Furthermore, alterations of the ontogenetic pattern in isozyme expression such as a delayed disappearance of the oocyte-coded LDH-B subunits or the enhanced expression of the embryonic *Ldh-2* gene during the early stage of embryonic development might enable a mouse embryo with lacking LDH-A activity to survive this critical phase of development. This might explain the very rare occurrence of homozygous mouse mutants detected among offspring derived from heterozygous LDH-A3N intercrosses due to an effect of genetic background, *e.g.*, by the loss of hybrid vigor.

The hypothesis that differences in LDH expression during mammalian embryogenesis determine lethality or viability of homozygous carriers of mutations leading to a complete LDH-A deficiency is supported by the comparison between mouse and humans. Despite the similar metabolic situation in early postimplantation embryos, human subjects with a complete absence of LDH-A subunits are fully viable (KANNO *et al.* 1980). This finding, which contrasts with the situation in the mouse, is explained by the predominance of LDH-B in the human fetus (FINE, KAPLAN and KUFTINEC 1963). In fact, among all mammalian species studied, mouse and man represent the extremes in the scale of percent LDH-B activity in early embryos (FIELDHOUSE AND MASTERS 1966). These findings emphasize the significance of the presence of isozymes and their tissue-specific and developmental pattern for the impact of a mutation on physiological functions.

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