A Mutation Affecting the Lactate Dehydrogenase Locus *Ldh-1* in the Mouse. II. Mechanism of the LDH-A Deficiency Associated With Hemolytic Anemia

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ABSTRACT

A procarbazine hydrochloride-induced mutation at the *Ldh-1* structural locus encoding the A subunit of lactate dehydrogenase (LDH) was used to study the molecular and metabolic basis of severe hemolytic anemia due to LDH-A deficiency in the mouse. The mutant allele designated *Ldh-1*$^{a\text{-}m1Neu}$ codes for an enzyme that as homotetramer differs from the wild-type enzyme by a marked instability, acidic shift of the pH profile, increased K_m for pyruvate and altered inhibition by high concentrations of this substrate. Except for the latter, all these altered properties of the mutant protein contribute to the diminished LDH activity in heterozygous and homozygous mutant individuals. Impaired energy metabolism of erythrocytes indicated by a relatively low ATP concentration is suggested to result in cell death at the end of the reticulocyte stage leading to the expression of hemolytic anemia with extreme reticulocytosis and hyperbilirubinemia. Despite the severe anemia, affected homozygous mutants exhibit approximately normal body weight and do not show noticeable impairment of viability or fertility. To date no such condition is observed in man. This discrepancy is likely due to the fact that in human erythrocytes both LDH-A and LDH-B subunits are expressed such that homozygotes for a LDH-A or LDH-B deficiency would not result in a comparably extreme LDH activity deficiency.

IN the mature erythrocyte glycolysis is the almost exclusive source of metabolic energy required for maintenance of cell function. Theoretically, any defect of the enzymes involved in this pathway severe enough to limit production of ATP should lead to premature erythrocyte death. It is therefore not surprising that in man inherited deficiency of most glycolytic enzymes has been found to be associated with nonspherocytic hemolytic anemia (DACIE 1985).

One exception appears to be lactate dehydrogenase (LDH, L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27). Neither the complete deficiency of the A nor of the B subunit of LDH in man (MARKERT 1968; MARKERT, SHAKLEE and WHITT 1975) results in increased hemolysis in affected individuals (MIWA *et al.* 1971; KANNO *et al.* 1980, 1988). The absence of deleterious effects to the erythrocyte seems to be also a feature of the first case of LDH deficiency described in *Mus musculus* (SOARES 1977, 1978). In contrast, a second murine LDH mutation detected by PRETSCH and CHARLES (1980) results in an extreme erythrocyte LDH deficiency as well as in a severe hemolytic syndrome in homozygous individuals (KREMER, DATTA and DÖRMER 1986; KREMER *et al.* 1987).

Although some data concerning genetic, biochemical and especially hematological characteristics of the mutation have been reported, up to now the molecular and metabolic aspects of the defect have not been elucidated. The fact that the mutation represents the first case of LDH deficiency associated with hemolytic anemia in a mammalian species, justified a further characterization to elaborate a clear-cut cause effect relationship between molecular anomalies, enzyme deficiency *in vivo*, erythrocyte dysfunction and pathophysiological syndromes.

MATERIALS AND METHODS

Animals: The original mutation was recovered as an electrophoretic variant in a screening program to detect newly occurring mutations in the offspring of mutagenically treated mice. A first description of the mutation given by PRETSCH and CHARLES (1980) and CHARLES and PRETSCH (1981) indicated that the mutation affects the *Ldh-1* structural locus. In concordance with the uniformity of nomenclature, the mutant allele originally designated *Ldh-1*^{c} is hereafter designated as *Ldh-1*$^{a\text{-}m1Neu}$ (allele short symbol *a-1N*).

Upon genetic confirmation of the mutation, it was backcrossed at least 10 generations to the C3H/El wild-type strain. For biochemical and physiological characterization, 10-week-old animals derived from *inter se* matings between heterozygotes were used. After weaning, four littermates 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*.

Sampling and preparation of tissues: Mice were weighed, anesthetized with ether and blood was collected through heparinized glass capillary tubes from the retroorbital sinus. Fresh blood was used for the determination of hematological parameters, glycolytic intermediates and met-

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abolic activities of erythrocytes as well as for the measurement of plasma compounds. Immediately thereafter, the mice were sacrificed by cervical dislocation and dissected. Organs were removed and weighed. The organo-somatic indices of liver, lung, kidneys, spleen and heart were calculated (organ weight × 100/body weight). To minimize any effects of circadian rhythm, the samples were taken between 8 and 10 a.m.

Preparations of blood, erythrocytes and tissues were performed as previously described (MERKLE and PRETSCH 1989; PRETSCH and MERKLE 1990).

Biochemical analysis: Enzymatic activity *in vitro* 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 constant (K_m) for pyruvate was performed using the standard assay for LDH with varying concentrations of pyruvate (0.1; 0.2; 0.3; 0.5; 1; 2; 5 mM). The apparent K_m was estimated by a least-squares regression fit.

Substrate specificity of LDH was tested by comparing the activity of the enzyme with 7 mM glyoxalate as substrate with that obtained in an assay containing 1 mM pyruvate as substrate.

Noncompetitive inhibition of LDH by oxalate was studied in the standard assay containing 1 mM pyruvate and 1 mM oxalate (Na salt). The inhibition of LDH by high concentrations of pyruvate was measured in the standard assay. All determinations of enzyme kinetic and inhibitory properties were performed with liver extracts at 25°. LDH activity levels in the assays of different genotypes were held constant by diluting the lysates of wild-type animals.

Stability of liver LDH was determined by incubating crude liver extract at 4° for 100 hr. Protein concentration in the extracts was approximately 20 mg/ml. At certain intervals aliquots were taken and diluted 1:51 with 0.15 M KCl. LDH activity was assayed immediately thereafter as described above.

pH dependence of LDH was obtained using a tris-glycine-phosphate buffer system (BEUTLER, MATHAI and SMITH 1968) with substrate concentrations of the standard assay.

Estimation of LDH activity *in vivo*: To estimate the enzyme deficiency *in vivo*, enzyme activities of wild type and mutant were calculated for a physiological pH of 7.4 and pyruvate concentrations measured in plasma of the respective genotype. Enzyme activities *in vitro* determined using the standard assay at pH 7.0 and pyruvate concentration of 8.5 mM were corrected for pH and effects of pyruvate concentration as follows:

$$\%LDH^*(m) = \%LDH \times \frac{K_m(w) \times I(w) \times [P](m) \times [LDH_{7.4}](m) \times [LDH_{7.0}](w)}{K_m(m) \times I(m) \times [P](w) \times [LDH_{7.0}](m) \times [LDH_{7.4}](w)}$$

where

[LDH*] is the estimated LDH activity *in vivo* determined by correcting LDH activity in the standard assay to pH 7.4 and plasma pyruvate concentrations,

[LDH] is the LDH activity *in vitro* measured using the standard assay

K_m is the Michaelis-constant for pyruvate

I is the percent LDH activity determined using the standard assay compared with V_{max}

[P] is the plasma pyruvate concentration (Table 5)

[$LDH_{7.4}$] is the LDH activity *in vitro* at pH 7.4 as percent of maximal enzyme activity

TABLE 1

***In vitro* LDH activity measured in blood, erythrocytes, plasma and several tissues from wild types, heterozygous and homozygous LDH-A-deficient mouse mutants**

	Ldh-1 genotype[a]		
	a/a	*a/a-1N*	*a-1N/a-1N*
Erythrocyte	100.0 ± 1.3 (192)	55.6 ± 1.4*	4.0 ± 0.5*
Blood	100.0 ± 1.2 (212)	56.2 ± 1.5*	6.2 ± 0.7*
Plasma	100.0 ± 10.2 (0.62)	88.2 ± 9.6	9.9 ± 1.8*
Liver	100.0 ± 1.6 (1,387)	88.7 ± 3.3*	47.7 ± 3.5*
Lung	100.0 ± 2.7 (596)	81.5 ± 2.9*	26.8 ± 1.4*
Kidney	100.0 ± 2.8 (1,387)	104.4 ± 4.2	90.5 ± 4.2
Spleen	100.0 ± 2.9 (751)	80.8 ± 2.9*	18.7 ± 1.7*
Heart	100.0 ± 2.7 (2,068)	98.2 ± 2.4*	81.0 ± 3.7*
Muscle	100.0 ± 2.2 (6,158)	90.5 ± 2.0*	41.5 ± 6.5*
Brain	100.0 ± 2.3 (1,208)	89.7 ± 2.0*	65.5 ± 1.8*

Data are expressed as percentage of LDH wild-type activity and are given as mean ± SEM of 14 animals. In parentheses the mean 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 were found in the amount of protein extracted per g tissue between mutants and wild types except for the lung where protein concentration in mutant homozygotes was 94.4 ± 2.5% of that of the wild type.

* Significant differences between wild types and mutants ($P \leq 0.05$).

[a] a = *Ldh-1*^{a}: wild-type allele; a-1N = *LDH-1*$^{a\text{-}m1Neu}$: LDH-A deficient allele.

[$LDH_{7.0}$] is the LDH activity *in vitro* at pH 7.0 as percent of maximal enzyme activity

(w) designates the wild-type genotype

(m) designates the mutant genotype.

Hematological data and plasma compounds: Hematological data, including erythrocyte number, hematocrit, hemoglobin concentration and osmotic fragility were obtained by standard techniques as previously described (MERKLE and PRETSCH 1989). Peripheral blood smears for determination of reticulocyte count were stained by mixing two parts of fresh blood with one part of 1% brilliant cresyl blue in isotonic saline. Measurement of total bilirubin in plasma followed the method of WAHLEFELD, HERZ and BERNT (1972). Plasma glucose was analyzed by the glucose-oxidase-peroxidase method (WERNER, REY and WIELINGER 1970). Determinations of pyruvate and lactate in plasma were performed as described by CZOK and LAMPRECHT (1974) and GUTMANN and WAHLEFELD (1974), respectively.

Glycolytic intermediates and adenine nucleotides in erythrocytes: Measurements of erythrocyte glycolytic intermediates and adenine nucleotides were done enzymatically according to MINAKAMI *et al.* (1965).

Metabolic activities of erythrocytes: Determinations of glucose consumption and release of lactate and pyruvate from erythrocytes in cell suspension at pH 7.4 and 37° were performed essentially according to the method of CHAPMAN *et al.* (1962).

TABLE 2

Physicochemical and kinetic properties of hepatic LDH of wild types, heterozygous and homozygous LDH-A-deficient mouse mutants

	Ldh-1 genotype[a]		
	a/a	*a/a-1N*	*a-1N/a-1N*
K_m (pyruvate) (mM)	0.218 ± 0.005	0.245 ± 0.008*	0.509 ± 0.025*
Pyruvate inhibition[b] (8.5 mM)	72.1 ± 1.5	91.0 ± 1.8*	98.4 ± 0.3*
(10 mM)	50.7 ± 1.0	85.4 ± 1.2*	99.7 ± 2.0*
Oxalate inhibition[c]	58.6 ± 2.2	67.8 ± 0.8*	83.5 ± 4.0*
Glyoxalate/pyruvate[d]	22.2 ± 0.8	22.4 ± 0.2	31.6 ± 1.9*
Stability[e]	99.5 ± 1.0	98.7 ± 0.8	38.5 ± 6.8*
Electrophoretic mobility	Normal	Normal	Normal
Isoelectric focusing pattern[f]	Normal	Altered	Altered
pH optimum	8.0–8.5	7.2–7.7	6.2–6.7
$[LDH_{pH\ 7.4}]$[g]	89.4 ± 1.5	99.3 ± 0.6*	88.3 ± 2.1
$[LDH_{pH\ 7.0}]$[h]	77.1 ± 1.3	93.4 ± 0.8*	94.8 ± 2.2*

Data are given as mean ± SEM of 6 animals (double determination).
* Significant differences between wild types and mutants ($P \leq 0.05$).
[a] Same symbols are used as in Table 1.
[b] Percent activity with 8.5 or 10 mM pyruvate as substrate compared with the maximal activity.
[c] Percent activity in the presence of 1 mM oxalate and 1 mM pyruvate compared with the activity without oxalate.
[d] Percent activity with 7 mM glyoxalate as substrate compared with the activity with 1 mM pyruvate as substrate.
[e] Percent remaining activity after 24-hr incubation at 4°.
[f] According to PRETSCH and CHARLES (1980).
[g] LDH activity *in vitro* at pH 7.0 as percent of maximal enzyme activity.
[h] LDH activity *in vitro* at pH 7.4 as percent of maximal enzyme activity.

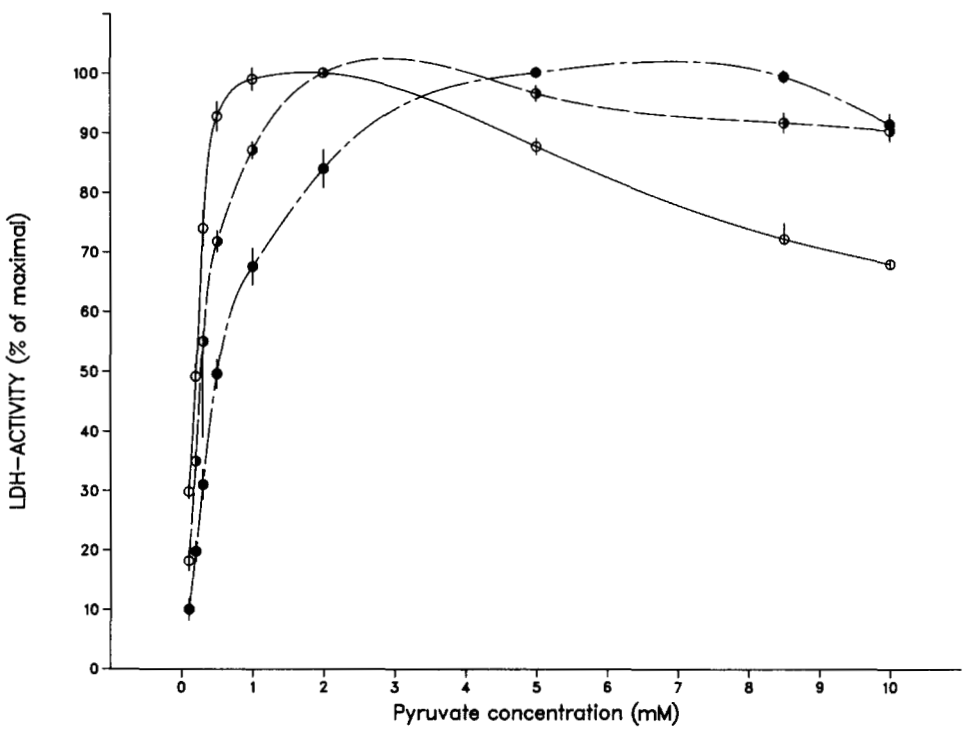

FIGURE 1.—Percent of maximal activity of liver lactate dehydrogenase of wild-type (○), heterozygous (◑) and homozygous (●) LDH-A-deficient mutants at different concentrations of pyruvate. Mean ± SEM of four animals (double determination).

Statistical analysis: In the biochemical and physiological characterization experiments, data from the approximately same number of female and male animals were used to determine the mean and standard error of the mean (SEM). For statistical comparisons between the different genotypes, Student's *t*-test was used.

RESULTS

Biochemical analysis: The results obtained by polyacrylamide gel electrophoresis and isoelectric focusing indicated that the mutation affects the LDH-A subunit (PRETSCH and CHARLES 1980; CHARLES and PRETSCH 1981). Analysis of physicochemical and kinetic properties of the mutant protein was therefore performed in liver, which, like the erythrocyte, expresses exclusively the LDH-A subunit but exhibits a considerably higher residual LDH activity than the red blood cell (Table 1). Physicochemical and kinetic properties of LDH-A in wild types, heterozygous and homozygous mutants are given in Table 2. The K_m of the mutant

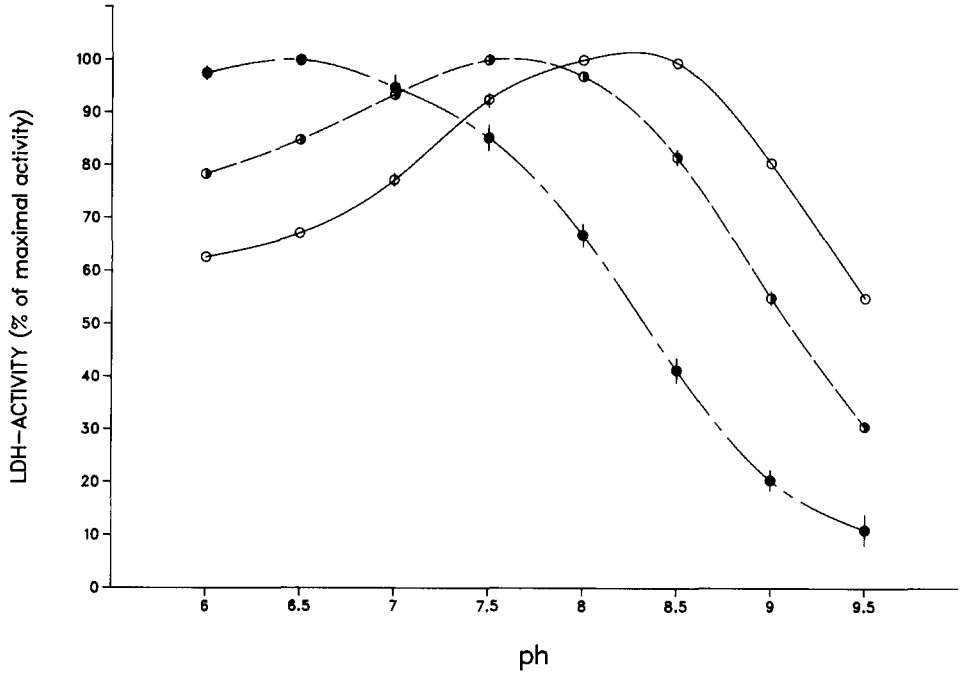

FIGURE 2.—Percent of maximal activity of liver lactate dehydrogenase of wild-type (○), heterozygous (◑) and homozygous (●) LDH-A-deficient mutants at different pHs. Mean ± SEM of four animals (double determination).

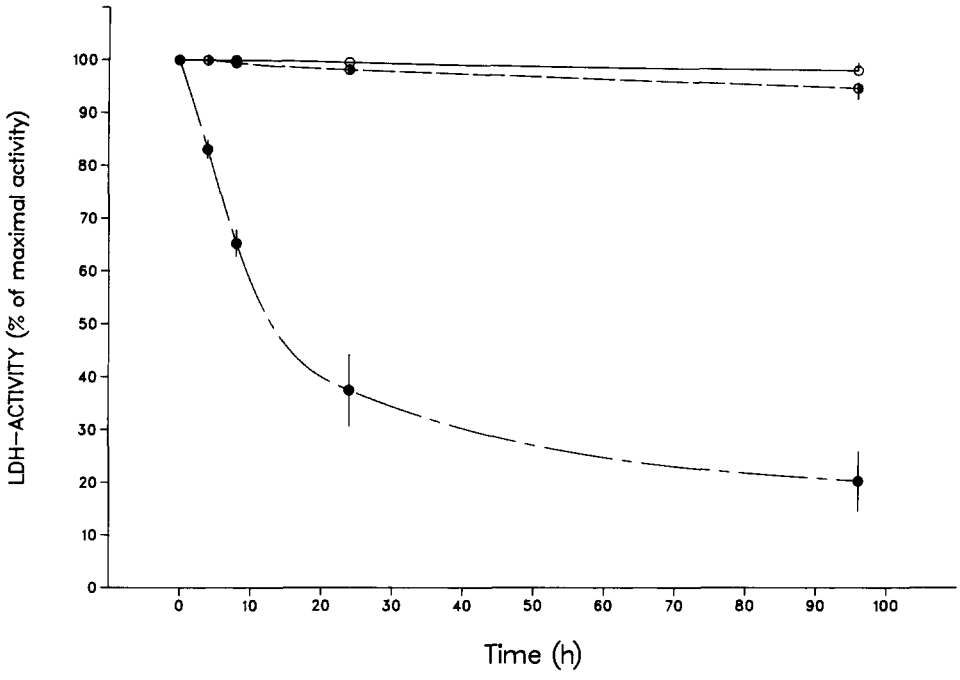

FIGURE 3.—Percent residual activity of liver lactate dehydrogenase of wild-type (○), heterozygous (◑) and homozygous (●) LDH-A-deficient mutants after incubation at 4°. Mean ± SEM of four animals (double determination).

LDH was slightly but significantly increased in heterozygotes and more than twofold elevated in homozygotes. Also maximal activity was reached at higher pyruvate concentrations in homozygous mutant LDH compared with the wild-type enzyme (Figure 1). Consistently, the inhibition of the enzyme with high pyruvate concentrations used, for example, in the standard assay was reduced for the mutant enzymes. Inhibition by oxalate was slightly reduced in heterozygotes but markedly decreased in homozygous mutants. Further, the relative utilization of glyoxalate compared with pyruvate was significantly increased only in mutant homozygotes. In addition, differences between mutant and wild-type enzyme were found in the pH dependence (Figure 2). There was a clear acidic shift of the pH profile by about 1.8 and 0.8 pH units, respectively, in homozygotes and heterozygotes.

Figure 3 depicts the relative loss of LDH activity in crude liver homogenate at 4°. Activity of wild-type LDH declined only minimally during the experimental period of about 100 hr. After 24 hr, no significant reduction in activity could be observed (Table 2). A remarkable instability was found for hepatic LDH of homozygous mutants. The enzyme lost about 60% of its initial activity after 24 hr, and approximately 80% after 100 hr. In contrast, heterozygotes showed

TABLE 3

Estimated *in vivo* LDH activity in erythrocytes, liver and spleen of wild types, heterozygous and homozygous LDH-A-deficient mouse mutants

	Ldh-1 genotype[a]		
	a/a	*a/a-1N*	*a-1N/a-1N*
Erythrocyte	100.0 (135)	37.8	1.6
Liver	100.0 (972)	60.4	19.6
Spleen	100.0 (526)	55.0	7.7

Data are expressed as percentage of LDH wild-type activity and calculated as described in MATERIALS AND METHODS. In parentheses the mean activity is given as units/g Hb in erythrocytes and in units/g protein in liver and spleen.

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

slightly stronger alteration than wild types. The difference in *in vitro* stability between wild types and heterozygous mutants is, however, more pronounced at higher temperatures (PRETSCH and CHARLES 1980).

LDH activity in blood, erythrocytes, plasma and tissues *in vitro*: Preliminary results of LDH activity measurements in various tissues have been reported by PRETSCH and CHARLES (1980). Because of the small sample size as well as the instability of the mutant protein, further detailed measurements were carried out in blood, erythrocytes, plasma and several tissues immediately after extraction (Table 1). As compared with the wild type, the enzyme activity level in erythrocytes of heterozygotes exhibited about 56% of the normal value, whereas homozygotes had approximately 4% residual activity. In other tissues the deficiency was less pronounced. This was especially true for heterozygotes where residual activities ranged between 80 and 100% of the wild-type level, depending on the tissue. In mutant homozygotes the lowest residual activity, about 20% of the wild-type level, was expressed in the spleen, followed by lung, muscle and liver. In heart and kidney residual activities amounted to 80 and 90% of the wild-type value, respectively.

Based on the markedly reduced stability characteristics of the mutant protein it is highly probable that a decreased intracellular stability of the mutant enzyme is responsible for the deficiency. However, the pattern of tissue specific activity seen in the three genotypes may be influenced by three main factors. The most important one is the tissue-specific expression of the two isozymic subunits A and B (FRITZ *et al.* 1969). Organs or cells in which the B subunit predominates showed the smallest decrease in activity whereas the main or exclusive occurrence of the A subunit correlates with a stronger deficiency in the respective tissue. However, in lung and muscle, which express a small percentage of B subunits, the deficiency was more pronounced than in liver tissue, which expresses exclusively LDH-A subunits. This discrepancy may indicate differential tissue-specific degradation rates for the mutant protein. Such differences in rates of intracellular protein degradation are well known for LDH (FRITZ *et al.* 1969, 1971). Finally, the presence or absence of protein synthesis, which would counterbalance the enhanced degradation rate, may be a further factor explaining tissue-specific differences in the degree of deficiency. This is especially true for mature erythrocytes in which no protein synthesis occurs (BENÖHR and WALLER 1975) as compared with liver in which protein synthesis takes place. As expected, the deficiency is more pronounced in erythrocytes. The discrepancy between erythrocytes and blood may be similarly explained, since white blood cells have the ability for protein synthesis and contribute about 10% of the total LDH activity in blood of wild types.

Estimated LDH activity in erythrocytes, liver and spleen *in vivo*: Since for the presented murine LDH deficiency the underlying mutation was found to be an intragenic event at the structural locus encoding LDH-A, the possible effects on the phenotype should be explainable by the reduced enzyme activity in the affected organism. However, measurements of LDH activity were performed using pyruvate concentrations far in excess of those occurring within cells and tissues. This is especially problematical since wild type and mutant enzyme were quite different in respect to the dependence of activity on substrate concentration and pH (Figures 1 and 2, Table 2) and consequently the *in vitro* measurements were not expected to exactly reflect the *in vivo* situation (KIRKMAN 1972). For a better approximation to the intracellular situation, the relative enzyme activities in exclusively LDH-A-containing cells and tissues such as erythrocytes, liver and spleen were calculated considering the different pH profiles and kinetic characteristics of LDH-A from wild types and mutants as well as the physiological pH and the intracellular pyruvate concentrations. Based on the free diffusion of pyruvate across cell membrane, for the latter the concentration in plasma was used (Table 5). As seen in Table 3, the estimated LDH deficiency for physiological conditions was far greater than that indicated by the *in vitro* measurements under standard assay conditions.

Glycolytic intermediates and metabolic activity of erythrocytes: Concentrations of glycolytic intermediates and adenine nucleotides, as well as glucose consumption, lactate formation and release of pyruvate into the incubation media were compared in mutant and wild-type erythrocytes to determine the effects of LDH-A deficiency on glycolytic flux and energy metabolism in these cells. The results are presented in Table 4. No significant differences could be found between erythrocytes from heterozygous and wild-

TABLE 4

Metabolic activity, glycolytic intermediates and adenine nucleotides in erythrocytes from wild types, heterozygous and homozygous LDH-A deficient mouse mutants

	Ldh-1 genotype[a]		
	a/a	*a/a-1N*	*a-1N/a-1N*
Glucose-6-phosphate	116 ± 6	118 ± 9	212 ± 8*
Fructose-6-phosphate	36 ± 2	37 ± 3	66 ± 3*
Fructose-1,6-diphosphate	48 ± 7	55 ± 3	4,780 ± 282*
Dihydroxyacetonephosphate	65 ± 6	71 ± 8	1,307 ± 92*
3-Phosphoglycerate	78 ± 8	74 ± 5	43 ± 6*
Pyruvate	173 ± 10	189 ± 5	289 ± 15*
Lactate	3,757 ± 273	3,898 ± 247	3,992 ± 373
Lactate/pyruvate	21.7 ± 1.7	20.6 ± 1.5	13.8 ± 1.4*
ATP	1,661 ± 71	1,710 ± 80	2,890 ± 109*
ADP	153 ± 8	151 ± 9	224 ± 13*
Glucose consumption	64.1 ± 4.3	63.9 ± 5.7	123.9 ± 12.6*
Lactate formation	118.5 ± 7.6	112.1 ± 3.8	92.0 ± 9.5*
Pyruvate formation	0	0	26.7 ± 3.5*

Data are given as mean ± SEM of 10 animals. Glycolytic intermediates and adenine nucleotides are expressed in nmol/ml blood cells. The levels of pyruvate and lactate are nmol/ml blood. Glucose consumption and formation of lactate and pyruvate is given in μmol/g Hb/hr.

* Significant differences between wild types and mutants ($P \leq 0.05$).

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

type individuals. In homozygous mutant erythrocytes, however, the assays for glycolytic intermediates revealed increased concentrations of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate (F1,6dP), and dihydroxyacetone-phosphate (DHAP) that were, respectively, 1.8, 1.8, 100, and 20 times greater than normal levels. Whereas the concentration of 3-phosphoglycerate (3-PG) was significantly reduced, the concentration of pyruvate was increased and that of lactate was unaltered, resulting in a markedly reduced lactate/pyruvate ratio. The concentrations of ATP and ADP also were elevated in erythrocytes from mutant homozygotes. Glucose consumption of homozygous mutant erythrocytes was increased twofold whereas lactate formation was significantly reduced. The release of pyruvate into the medium, which was not detectable in wild-type and heterozygous erythrocytes, was present in homozygous mutant erythrocytes and amounted to about one third of the rate of lactate formation.

The interpretation of studies of erythrocytes from anemic mice is generally complicated by the altered composition of the erythrocyte population with a large representation of reticulocytes. There is evidence that during aging the erythrocyte undergoes important alterations in both composition and metabolic capacities. This includes a decline of glycolysis and glycolytic intermediates (CHAPMAN and SCHAUMBURG 1967; SEAMAN, WYSS and PIOMELLI 1980). Thus, it may be expected that the young age of the reticulocyte-rich erythrocyte population observed in homozygous mutants is a factor contributing to the elevated concentrations of glycolytic intermediates in erythrocytes of these animals. The marked elevation of F1,6dP and DHAP as well as the reduced levels of 3-PG are not explainable by aging effects but indicate a retarded glycolytic flux at the step of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulting in an accumulation of intermediary substrates before the enzyme block and a decrease behind it. In addition, the reduced lactate/pyruvate ratio and the abnormal pyruvate formation of homozygous mutant erythrocytes indicated a further block at the LDH step. Since in contrast to the latter the apparent block at the GAPDH step did not correspond to a reduction of the activity of the respective enzyme, it is suggested that it is a secondary consequence of the reduced LDH reaction mediated by a deficiency of coenzyme. Reduced glycolytic flux and resulting impaired energy metabolism are furthermore indicated by a glucose consumption and ATP concentration in mutant erythrocytes, which in comparison to wild-type cells are elevated but which are lower than that in murine red-cell populations with comparable reticulocyte count (HUTTON and BERNSTEIN 1973; BERNSTEIN 1980).

Hematological data: As already described by KREMER *et al.* (1987) homozygous mutants exhibited a marked decrease in the erythrocyte number, hematocrit and in the concentration of hemoglobin, whereas heterozygous animals did not differ significantly from the wild type. The elevation in mean cellular volume as well as the reduction of mean cellular hemoglobin concentration of homozygous mutant erythrocytes compared with those of the wild types reflected the extreme reticulocytosis (ca. 90%) in these animals. In comparison with erythrocytes

TABLE 5

Several plasma compounds of wild types, heterozygous and homozygous LDH-A deficient mouse mutants

	Ldh-1 genotype[a]		
	a/a	*a/a-1N*	*a-1N/a-1N*
Total bilirubin (mg/dl)	0.38 ± 0.05	0.39 ± 0.03	1.76 ± 0.21*
Glucose (mg/dl)	126.2 ± 4.8	127.1 ± 3.0	128.0 ± 4.2
Pyruvate (mM/l)	0.19 ± 0.02	0.20 ± 0.02	0.31 ± 0.02*
Lactate (mM/l)	3.91 ± 0.42	3.99 ± 0.33	4.02 ± 0.32
Lactate/pyruvate	20.6 ± 1.2	20.0 ± 1.8	13.0 ± 1.1*

Data are given as mean ± SEM of 12 animals.
* Significant differences between wild type and mutants ($P \leq 0.05$).
[a] Same symbols are used as in Table 1.

from wild types and heterozygotes that showed no significant differences, homozygous mutant erythrocytes revealed a flattened osmotic fragility curve with an additional shift toward lower NaCl concentrations. This finding indicated that the macrocytic erythrocytes from mutant homozygotes were more resistant to osmotic stress in hypotonic media than the normocytes of wild types. Since macrocytic young cells from a normal human erythrocyte population generally are also more resistant to hypotonic salt solutions than older cells (MARKS and JOHNSON 1958), the alteration of osmotic fragility curve of mutant homozygotes is likely a result of reticulocytosis than to be a direct consequence of the enzyme defect in the erythrocyte.

Total bilirubin and several glycolytic metabolites in plasma: Table 5 summarizes the results of the determination of total bilirubin, glucose, pyruvate and lactate in plasma. There were no significant differences between heterozygotes and wild types for all parameters studied. In accordance with the enhanced loss and reduced life-span of erythrocytes (KREMER, DATTA and DÖRMER 1986), the concentration of total bilirubin in plasma from mutant homozygotes was markedly increased. Pyruvate concentration in plasma was significantly elevated whereas the levels of glucose and lactate exhibited normal values. The first was suggested to be a consequence of the abnormal pyruvate formation observed in homozygous mutant erythrocytes (Table 5).

Body weight, organo-somatic indices: Compared with wild types, heterozygotes showed normal values for all parameters studied. Normal values were also observed for body weight and the kidney-somatic index of mutant homozygotes. In contrast, somatic indices of liver, lung and heart in these animals were increased by, respectively, 6.1, 8.1 and 17.5% of the wild-type value. The most prominent increase, however, affected the spleen, which was increased approximately eightfold in homozygous mutants compared with wild types.

Viability of heterozygous and homozygous mutants: When progeny of heterozygous and wild-type animals were classified at 4–6 weeks of age, homozygous wild-type and heterozygous mutant offspring were found in a ratio of approximately 1:1. This suggested that heterozygotes are fully viable. A similar conclusion may be drawn for homozygous mutants, which occurred in crosses between heterozygotes and were found to be in agreement with that expected. Since in crosses between homozygous mutants *inter se* no significant reduction of litter size could be observed, the homozygous mutants seem also to be fully viable and fertile.

DISCUSSION

The present investigation focused on several aspects of LDH-A deficiency in the mouse that are useful in understanding the relationship between genotype and phenotype, *i.e.*, between the mutational event and resulting physiological or morphological alterations. These aspects include site and nature of the underlying mutation, molecular mechanism and severity of the deficiency *in vivo*, as well as metabolic effects in the erythrocyte and physiological alterations in affected individuals. Furthermore, results contribute to an understanding of species differences for LDH deficiency in humans and mice.

Nature of the mutation: Based on the results of the electrophoretic studies performed in several tissues, CHARLES and PRETSCH (1981) suggested that the underlying mutation is a structural mutation affecting the *Ldh-1* locus. This was genetically confirmed by linkage analysis (PRETSCH 1989). The results of the electrophoretic, kinetic and stability studies show that the mutation changes not only the net charge of the mutant protein but also leads to conformational alterations affecting the active site of the enzyme, simultaneously reducing its stability. This finding, in turn, clearly suggests a base-pair change in the DNA sequence of the *Ldh-1* structural gene.

Molecular mechanism of the deficiency: One of the most prominent alterations of the mutant enzyme is its reduced stability *in vitro*. Among all thus far described murine LDH-A mutants (SOARES 1978; MERKLE *et al.* 1992; MERKLE and PRETSCH 1992), it is the most unstable protein. Although studies dealing with enzyme-deficient mouse mutants exemplified that distinct thermostability characteristics of an enzyme *in vitro* are not in every case correlated with its stability *in vivo* (MERKLE and PRETSCH 1992), it is highly probable that an enhanced intracellular inactivation due to a pronounced instability is the main cause for the enzyme deficiency. This is supported by

the fact that among cells and tissues with exclusive LDH-A expression the deficiency is most pronounced in erythrocytes that lose the ability of counterbalancing protein synthesis beyond the reticulocyte stage. However, altered kinetic characteristics as well as a changed pH profile of the mutant enzyme are further factors contributing to enzyme deficiency. In fact, correcting the standard *in vitro* assay measurements to physiological pH and substrate levels, a more pronounced deficiency *in vivo* is predicted.

Metabolic effects of LDH deficiency: The metabolic role of LDH as the final enzyme in anaerobic glycolysis, its role as lactate-oxidizing catalyst in hepatic and renal gluconeogenesis, as well as in utilization of lactate for production of metabolic energy in the mammalian heart is well understood (VESELL 1975). In mature erythrocytes and other anaerobic cells in which glycolysis is the major energy delivering pathway, the primary function of LDH is to reoxidize NADH formed by the GAPDH reaction, thereby providing the intracellular NAD^+/NADH ratio necessary for glycolysis to continue (BENÖHR and WALLER 1975). Therefore, JAFFÉ (1970) assumed that in LDH deficiency the accumulation of NADH and the relative deficiency of NAD^+ might result in indirect inhibition of glycolysis whereas the accumulated pyruvate might diffuse out of the cell without producing deleterious effects. This prediction was confirmed by findings in LDH-deficient humans. Individuals with LDH deficiency due to a complete absence of A subunits showed impaired glycolysis and reduced production of ATP in muscle (KANNO *et al.* 1988). Increased concentrations of glyceraldehyde-3-phosphate, DHAP and F1,6dP, in fact, indicated a block of metabolic flux at the GAPDH step. Elevated concentrations of pyruvate and unaltered concentrations of lactate in plasma after ischemic forearm work pointed to an impaired conversion of pyruvate to lactate and an increased diffusion of pyruvate out of the cell. Similar alterations of glycolytic intermediates were observed also in erythrocytes from LDH-B deficient humans (MIWA *et al.* 1971; KANNO *et al.* 1983) and in erythrocytes and plasma from homozygous mouse mutants of the present study. The metabolic mechanism of severe LDH deficiency in erythrocytes thus may be summarized as follows: The primary block in the conversion of pyruvate to lactate leads to an accumulation of pyruvate and an abnormal release of pyruvate from affected erythrocytes resulting in a decreased lactate/pyruvate ratio in plasma. The defective NAD^+ regeneration due to the reduced pyruvate to lactate conversion generates a retarded glycolytic flux at the GAPDH step resulting in the corresponding alterations of intracellular metabolite concentrations including a reduced ATP production. The impaired energy metabolism results in cell death of erythrocytes.

The extremely short life span of homozygous mutant erythrocytes estimated to be about 2 days (KREMER, DATTA and DÖRMER 1986), however, suggests that the deleterious effects take full effect first at the end or beyond the reticulocyte stage. This might be attributed to the almost complete and more variable biosynthetic and metabolic capacities of the reticulocyte compared with the mature red blood cell (BENÖHR and WALLER 1975). First, the reticulocyte provides its energy essentially by complete glucose oxidation via glycolysis and citrate cycle where parts of the glycolytic NAD^+ requirement are provided by mitochondrial oxidative processes. Moreover, it is able to utilize as energy substrate not only glucose but any material that enters the citrate cycle such as amino acids or acetyl Co A derived from fatty acids. Second, the reticulocyte's capability of protein synthesis provides constant generation of newly synthesized enzyme molecules. An enzyme deficiency due to unstable proteins should therefore be less pronounced in the reticulocyte than in more mature erythrocytes where protein synthesis is absent.

It must be emphasized that the easily detectable metabolic alterations were only detected in erythrocytes from homozygous mutants. The calculated residual *in vivo* LDH activities for wild types, heterozygotes and mutant homozygotes are approximately 135, 51 and 2 IU/g Hb. The rate of lactate formation of the normal erythrocyte is approximately 2 mmol/min/g Hb. Thus, only the estimated LDH activity of erythrocytes from homozygous mutants falls within a range where it is theoretically rate limiting for the overall flux of glycolysis. From this point of view, it is estimated that in murine erythrocytes LDH deficiency must exceed 95% of the wild-type level to interfere markedly with metabolic flux to cause pathophysiological effects.

Physiological effects: Consistent with the absence of easily detectable metabolic alterations in erythrocytes from heterozygotes, heterozygotes showed no significant physiological or morphological differences compared with the wild type. On the contrary, the metabolic dysfunctions in homozygous mutant erythrocytes are so severe that they ultimately lead to the rapid destruction of the cell. Despite the immense red blood cell loss, reflected in a pronounced hyperbilirubinemia, hemoglobin could be maintained at 50–60% of that of the normal level. The efficient compensation for the continuous hemolytic erythrocyte loss was recently found to be a result of a 10- to 50-fold expansion of erythropoiesis primarily in spleen (KREMER *et al.* 1987). Together with the increased splenic erythrocyte sequestration activity (DATTA *et al.* 1988) the enhanced erythropoiesis is reflected in

an enormous splenic enlargement. Data from anemic humans (DACIE 1985) suggest that processes involved in the elevated erythropoiesis and sequestration activity are also responsible for hepatomegaly found in anemic LDH-deficient mice. Besides replenishment of defective erythrocytes, the increase of cardiopulmonary function seems to be a further detectable phenomenon in these animals. The increased relative size of lung and heart suggests that the reduced oxygen transporting capacity of blood is compensated for by an increased respiratory function and by an enhanced cardiac index. Thus, both hepatosplenomegaly as well as hypertrophy of lung and heart may be interpreted as adaptive responses to hypoxic conditions of anemia and consequently as indirect, secondary manifestations of the mutation. Normal body weights and no noticeably impaired viability or fertility of these mutants suggest that exclusively erythrocytes are directly affected by the mutation. This restriction of the primary physiological effect of the mutation to the erythrocyte is due to the fact that the main molecular mechanism of the enzyme defect is an increased instability of the mutant protein.

Moreover, it must be noted that normal body weight and viability of anemic LDH-deficient mice contrasts with the pathological feature of mice affected by severe, spherocytic hemolytic anemia due to a defect in the erythrocytic membrane protein, spectrin. These animals have a reduced probability to reach adulthood and as young adults are much smaller than their normal littermates (UNGER *et al.* 1983). The cause of this discrepancy is not clear. However, the comparison of hemoglobin levels and spleen sizes indicates that anemia due to spectrin deficiency is more severe than that caused by LDH deficiency, suggesting that the severity of anemia might play a role.

Physiological effects of LDH deficiency in humans: As already mentioned, severe LDH deficiency as a result of a complete lack of either the A or the B subunit has not been documented in humans. Based on the tissue-specific subunit expression in the human tissues (VESELL and BEARN 1962; ENGEL, KREUTZ and WOLF 1972) complete LDH-A deficiency resulted in an only slight decrease of enzyme activity in erythrocytes of affected individuals whereas the reduction of LDH activity to approximately 5% of normal was extreme in skeletal muscle (KANNO *et al.* 1980). Consistently, the affected individuals exhibited pathophysiological symptoms in the muscle but did not show any signs of hemolytic anemia (KANNO *et al.* 1988). Interestingly, except for some abnormalities in erythrocytic glycolytic intermediates, there was also no evidence for hemolysis in LDH-B-deficient individuals with an approximate 5% residual LDH activity in erythrocytes (MIWA *et al.* 1971). This surprising absence of severe metabolic dysfunctions in erythrocytes having such an extreme LDH deficiency has been postulated by KANNO *et al.* (1983) as due to the operation of NADH methemoglobin reductase, a further specific NADH reoxidizing system, which compensates for the deficient LDH reaction.

In light of the pathophysiological consequences of severe LDH deficiency in the mouse that also have methemoglobin reductase, this interpretation seems not to be sufficient. The comparison between residual LDH activity in LDH-B-deficient human erythrocytes of about 9.9 IU/g Hb (DATTA *et al.* 1988) with that in erythrocytes from homozygous mouse mutants indicates that the deficiency is more severe in the murine than in the human erythrocytes. Because glycolytic rates of normal murine erythrocytes were found to be similar to that of human erythrocytes (CHAPMAN *et al.* 1962) it might be that the residual activity in LDH-B-deficient human erythrocytes is sufficient to provide a considerable part of the NAD^+ required for glycolysis. The expression of both LDH isozymes in human erythrocyte would maintain the minimally required LDH rest activity even in homozygous deficiency mutants. The comparable situation in human erythrocyte to the homozygous LDH-A murine mutation could only be achieved by a double homozygote deficient in both LDH-A and LDH-B. Since expression of both A and B subunits in erythrocytes is common in most mammals except, for example, rodent species of the suborder Myomorpha including *Mus musculus* (ENGEL, KREUTZ and WOLF 1972), it would be predicted based on the present results that hemolytic anemia due to LDH deficiency would only result when the residual LDH activity is extremely low. This is only probable for those mammalian species that express a single LDH structural locus.

In conclusion, our results elucidate the dependence of pathophysiological effects on the type of enzyme deficiency mutation as well as the tissue-specific expression pattern and tissue-specific metabolism.

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