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# Lactate Dehydrogenase Isoenzyme Electrophoretic Pattern in Serum and Tissues of Mammalian and Bird Origin

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## Abstract

Lactate dehydrogenase (LDH) is a tetrameric enzyme that in vertebrates exists in five electrophoretically distinguishable forms known as isoenzymes. According to their different mobility to anode, they are denoted LDH<sub>1</sub> (H<sub>4</sub>), LDH<sub>2</sub> (H<sub>3</sub>M), LDH<sub>3</sub> (H<sub>2</sub>M<sub>2</sub>), LDH<sub>4</sub> (HM<sub>3</sub>), and LDH<sub>5</sub> (M<sub>4</sub>). A buffer system of the pH values 8.6–8.8 is commonly used for the separation of these isoenzymes in mammals. In the case of bird LDHs, the observation of five fractions is very difficult under this condition as they usually produce a pattern of one diffuse zone. Isoelectric focusing technique (IEF) in the pH range of 3–9 enabled a good and clear resolution of all five bird LDHs. Using this technique, it was also possible to observe the pattern in some tissues of chicken embryo.

**Keywords:** lactate dehydrogenase isoenzymes, isoelectric focusing technique, birds, mammals, embryo

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## 1. Introduction

Lactate dehydrogenase (EC 1.1.1.27, LDH) is an enzyme ubiquitarily distributed in cells of living systems. It catalyzes an interconversion of lactate and pyruvate with nicotinamide adenine dinucleotide (NAD<sup>+</sup>) as coenzyme both in cytoplasm and in mitochondria [1, 2]. This enzyme is also present in the sera of animals and humans where its changed pattern serves as a valuable adjunct indicator in the diagnostics of heart, skeletal muscle, and liver disorders [3]. In tissues of vertebrates, LDH exists in several electrophoretically distinguishable forms known

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as isoenzymes. They catalyze the same biochemical reaction but differ from each other in their kinetic characteristics ( $K_m$ ,  $k_{cat}$ ), physicochemical properties (different net charge), response to the inhibition by substrate (pyruvate) [4–6], and immunological response. Despite a profound understanding of LDHs, there was a lack of data concerning their relative distribution and pattern in bird tissues and sera. This gap in our knowledge of these enzymes was probably due to the fact that electrophoretic techniques routinely used in separating mammalian LDH isoenzymes did not produce a satisfactory separation of all bird isoenzymes [7, 8]. Thus, their application in research as well as in diagnostic practice remained in birds limited to the determination of the total LDH activity without the possibility for the correct interpretation of data in relation to their quantitative patterns in respective tissues/organs. The aim of our work was to separate lactate dehydrogenase isoenzymes of bird origin and to characterize the pattern of their relative distribution in tissues and sera using a suitable electrophoretic technique.

## 2. Isoenzymes of lactate dehydrogenase and their electrophoretic separation

LDH is a tetrameric enzyme that exists in three basic homotetrameric forms in vertebrates:  $H_4$  ( $LDH_1$ ),  $M_4$  ( $LDH_5$ ), and  $C_4$  ( $LDH-X$ ) [9–16]. Three structurally different polypeptide chains of homotetrameric LDH molecules are encoded by three different genes [13, 17–19]. Lactate dehydrogenase  $H_4$  and  $M_4$  forms, also called somatic LDH [20], are present in tissues of all studied vertebrates, while the tissue distribution of  $LDH-C_4$  isoenzyme varies from organism to organism. In mammals and columbid birds, this isoenzyme is expressed only in mature testes [19, 21, 22]. Except  $H_4$  and  $M_4$  homotetramers, somatic LDH also exists in three hybrid forms, thus being present in five structural entities in cells of most tissues. According to their different motility to the anode, somatic LDHs are denoted  $LDH_1$  ( $H_4$ ),  $LDH_2$  ( $H_3M$ ),  $LDH_3$  ( $H_2M_2$ ),  $LDH_4$  ( $HM_3$ ), and  $LDH_5$  ( $M_4$ ) with  $LDH_1$  having the highest and  $LDH_5$  the lowest migration rate to the anode. Three hybrid forms ( $LDH_2$ ,  $LDH_3$ , and  $LDH_4$ ) are relatively equally spread between  $LDH_1$  and  $LDH_5$  on the electrophoreogram. Protomers of the somatic LDH isoenzymes are, in general, designated H (heart) or B, and M (muscle) or A according to the domination of homotetrameric molecules in the cells of respective organs of adult vertebrates. Hybrid forms of LDH are present in various levels in the individual organs of an animal/human.

Although catalyzing the same overall reaction, LDH isoenzymes differ in their kinetic characteristics ( $K_m$ ,  $k_{cat}$ ) as documented in **Table 1** [4–6, 23–25].

As seen,  $H_4$  homotetramers have a lower value of Michaelis constant  $K_m$  for pyruvate as substrate. Moreover,  $H_4$  is more sensitive to inhibition by high pyruvate concentration than  $M_4$  isoenzyme, which is relatively indifferent to substrate concentration [3].

Different structures of lactate dehydrogenase isoenzyme molecules predetermine their different net charge and, consequently, different migration rate in electric field. It is known that the charge of a protein and, therefore, its motility in the electric field vary with the pH of its environment. Maximum resolution is achieved when the proteins of interest have

|                              |                | Chicken              | Rabbit               | Cattle               |
|------------------------------|----------------|----------------------|----------------------|----------------------|
| $K_m$ (mol.l <sup>-1</sup> ) | H <sub>4</sub> | $8.9 \times 10^{-5}$ | $6.7 \times 10^{-5}$ | $7.1 \times 10^{-5}$ |
|                              | M <sub>4</sub> | $3.2 \times 10^{-3}$ | $3.5 \times 10^{-4}$ | $1.0 \times 10^{-4}$ |
| $k_{cat}$ (s <sup>-1</sup> ) | H <sub>4</sub> | 45,000               | 41,000               | 49,400               |
|                              | M <sub>4</sub> | 93,400               | not determined       | 80,200               |

**Table 1.** Kinetic constants determined for the catalytic action of H<sub>4</sub> and M<sub>4</sub> homotetramers of lactate dehydrogenase.

widely varying electrical charges. Conversely, no or poor separation of proteins is achieved when there are only small differences in the electrical charges of molecules at a given pH values. Changing the pH of the electrophoresis media may alter the charges on respective proteins, thus producing a better separation. Generally, a buffer system of pH 8.6 is chosen for the separation of the five lactate dehydrogenase isoenzymes. At pH 8.6, their electrophoretic migration depends on the two pure types, that is, the H<sub>4</sub> and M<sub>4</sub> forms [9]. The more widely the two homotetramers differ in charge (the case of mammalian lactate dehydrogenases), the more separable are the hybrids by electrophoresis. In the case of bird lactate dehydrogenases, the two pure types migrate relatively close together toward the negative pole at pH 8.6, and the observation of the hybrid forms under these conditions is very difficult [9]. To overcome this difficulty, we chose a gradient of pH 3–9 for the electrophoretic separation of bird lactate dehydrogenase isoenzymes (isoelectric focusing technique, IEF) [8]. By this technique, we achieved a good and clear resolution of all five forms of the enzyme in serum and tissues of chicken (adult as well as in embryonic), turkey, pheasant, and pigeon. We also compared patterns in bird tissues and sera with those of mammalian origin.

### 3. Preparation of the samples for electrophoresis

Sera from various animal species were obtained from rested and clinically healthy animals. The blood collection for the serum samples was performed by standard procedures, and no hemolyzed sera were used for the examination. The determination of total lactate dehydrogenase activity as well as separation of the isoenzymes was performed on the same day as the blood collection.

Tissue samples were taken from birds after decapitation and immediately put into ice-cold buffer saline. The tissues were cut into small pieces and washed in buffered saline to remove excess blood and connective tissue. Two grams of tissue were homogenized in 10 volumes of cold buffer (0.05 mol/L Tris-HCl buffer, pH 7.3 with 0.01% EDTA). The homogenate was centrifuged at  $19,000 \times g$  for 60 min at 4°C, and the supernatant was used for enzyme assay and electrophoretic separation.

Blood for erythrocytes LDH pattern was collected in test tubes containing sodium heparin and centrifuged at  $450 \times g$  for 10 min at 4°C. Plasma was discarded and erythrocytes washed

thoroughly three times in twice their volume, and the supernatant discarded. The cells were then lysed with the addition of three volumes of cold distilled water and centrifuged at  $19,000 \times g$  for 60 min at  $4^{\circ}\text{C}$ .

Chicken embryonic organs/tissues were taken from 9-day-old chicken embryo ( $n = 6$ ), washed in buffer saline, and homogenated in 50 mM Tris-HCl, pH 7.5, with the content of 0.01% EDTA using Precellys 24—Dual homogenizer (Bertin Technologies, France). The homogenates were centrifuged at  $25,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , and the supernatants served as the source of the enzyme.

Catalytic activity of LDH was assayed colorimetrically at  $37^{\circ}\text{C}$  using lactate as the substrate with  $\text{NAD}^+$  as the coenzyme and brownish-red pyruvate hydrazone as the measured product ( $A_{505}$ ). Protein concentration for the calculation of specific enzyme activity (U/g) was determined using Bradford method [26]. The activity of LDH in erythrocytes was expressed in enzyme units per gram of hemoglobin (U/g of hemoglobin).

#### 4. Electrophoretic techniques used for separation of LDH isoenzymes

Lactate dehydrogenase isoenzymes can be separated using various supporting media such as starch, agarose, cellulose acetate, and polyacrylamide gel. The separations should be carried out with cooling to  $4^{\circ}\text{C}$  in order to prevent the possibility of enzyme inactivation, particularly heat-sensitive slow-moving isoenzymes ( $\text{LDH}_4$  and  $\text{LDH}_5$ ) [3].

Two types of polyacrylamide gel electrophoresis for the separation of lactate dehydrogenase isoenzymes of mammalian and bird origin were used in our laboratory (PhastSystem, Pharmacia LKB, Sweden):

1. Gradient polyacrylamide gel electrophoresis with continuous 10–15% gradient gel zone and 2% cross-linking (PAGE 10–15) as well as with continuous 8–25% gradient gel zone (PAGE 8–25). Separation conditions: 400 V, 10.0 mA, 45 min separation time at  $4^{\circ}\text{C}$  and with a buffer system of 0.88 mol/L L-alanine/0.25 mol/L Tris pH 8.8.
2. Isoelectric focusing technique with a pH range of 3–9 in homogeneous 5% polyacrylamide gel containing Pharmalyte carrier ampholytes (IEF 3–9). Separation conditions: 2000 V, 2.5 mA,  $15^{\circ}\text{C}$ , 20 min separation time.

#### 5. Detection and quantification of lactate dehydrogenase isoenzymes

As many proteins were separated by electrophoresis, lactate dehydrogenase isoenzymes were detected (stained) specifically using a colorimetric method with an assay system in 0.1 M Gly-NaCl-NaOH, pH 8.3: 1.0 mol/l sodium lactate (1.0 ml), 10 mg/ml  $\text{NAD}^+$  (1.5 ml), 1.0 mg/ml nitro blue tetrazolium (NBT) (6.0 ml), and 2.0 mg/ml phenazine methosulfate (PSM) (0.6 ml)

up to 30 ml with 0.1 M Gly-NaCl-NaOH, pH 8.3. In this reaction mixture, the gels were incubated at 37°C for 20–30 min. Then, they were immersed into 7.5% (v/v) acetic acid to stop the reaction. To detect lactate dehydrogenase isoenzyme zones on the electrophoreogram, we avoid using Tris-HCl buffer as it produced quite an intensive background on the gel. PhastImage system (Pharmacia LKB, Sweden) served for densitometric scanning (613 nm) of the pattern, and for the determination of relative distribution (%) of the isoenzyme fractions, GEL LOGIC 100 IMAGING SYSTEM with Kodak 1D Image Analysis Software (Japan) was used as well. To identify individual bird LDH isoenzymes on the electrophoreogram, a principle commonly used for the identification of mammalian LDH isoenzymes was applied: the fraction nearest to the anode was designated LDH<sub>1</sub> and that nearest to the cathode LDH<sub>5</sub>.

### 6. Patterns of LDH isoenzymes and their interpretation

Four to five isoenzymes of LDH are usually present in normal sera of animal and human beings as a result of natural degradation of cells of various tissues/organs. Their quantitative distribution in the serum is different and relatively characteristic for a particular biological species (Figure 1) [8]. After their separation in a concentration gradient of polyacrylamide (10–15%) and at pH 8.8, mammalian lactate dehydrogenases were separated with a good resolution, whereas bird serum (chicken) produced only one, somewhat diffuse enzymatic zone [8].

A good and clear resolution of bird LDH (chicken and pheasant) isoenzymes with all five isoenzymes zones was achieved using isoelectric focusing technique in a pH range of 3–9 [8]. A similar pattern was also produced by turkey isoenzymes [27]. A comparison of LDH catalytic activity and relative distribution (%) of its isoenzymes in the sera of the investigated animals (Figure 1) revealed that the predominant portion of chicken serum LDH activity was concentrated in the muscle form of the enzyme (LDH<sub>5</sub>) (66%), followed by LDH<sub>4</sub> (23%). LDH<sub>1</sub> to

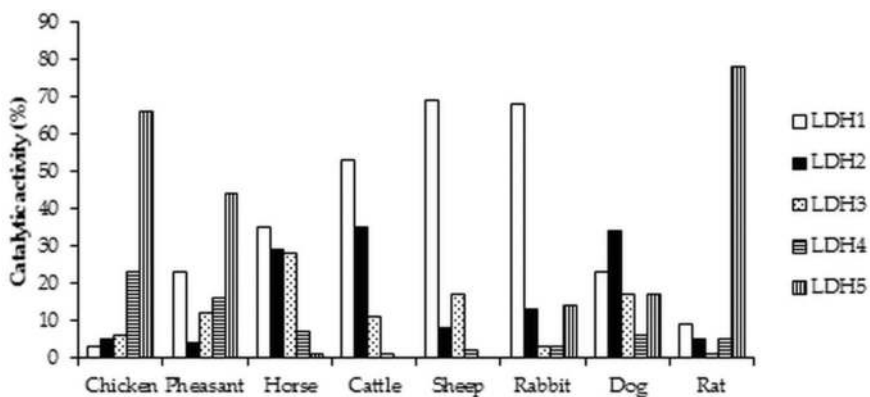


Figure 1. Lactate dehydrogenase isoenzyme patterns in the serum of some birds and mammals.

LDH<sub>3</sub> occurred in low amounts with fairly similar proportions (3–6%). The muscle isoenzyme was also the main fraction in pheasant and turkey serum [8, 27] (44 and 50%, respectively). Heart homotetramer as well as hybrid forms were present in smaller amounts. Most mammals showed a reverse pattern: the main portion of serum lactate dehydrogenase activity migrated in the first three anodic fractions, while LDH<sub>4</sub> and LDH<sub>5</sub> made only minor contributions (**Figure 1**). The serum pattern of LDH isoenzymes probably reflects the pattern of the main LDH organ donors (heart, skeletal muscle, and liver) and differs from animal to animal species. As an example, the quantitative analysis can serve organ/tissue pattern and relative distribution of LDH isoenzymes in chicken (**Figure 2**) [28]. The patterns of lactate dehydrogenase isoenzymes in various chicken tissues, erythrocytes, and serum can be divided into three groups: (1) those with cathodic domination, (2) those with anodic domination, and (3)

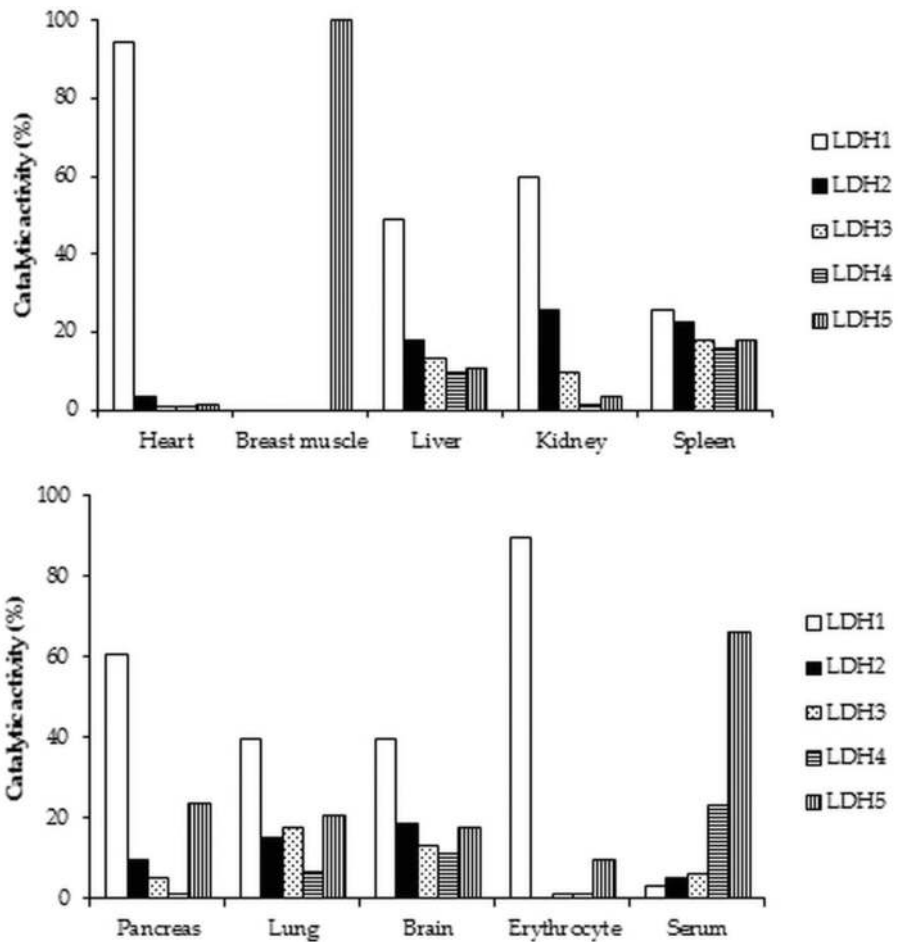


Figure 2. Lactate dehydrogenase isoenzymes pattern in chicken tissues and blood.

those distributed in the entire electrophoreogram. The anodic domination (LDH<sub>1</sub>) is seen in the cardiac muscle and erythrocytes, while the cathodic domination (LDH<sub>5</sub>) is seen in the breast muscle. Other tissues and serum exhibit a more spread pattern with some anodic tendency (kidney, liver), cathodic tendency (skeletal muscle, serum), and more even distribution (spleen, pancreas, lung, and brain). Similar distribution and pattern of LDH isoenzymes were described in turkey [27].

Comparing the isoenzyme patterns of birds (chicken, turkey) [27, 28] to human and other mammals reveals some differences. While the heart and skeletal muscle patterns are similar in birds and mammals, it differs in other tissues, especially in the liver. While in most mammals, the liver pattern is quantitatively dominated by the slower moving cathodic isoenzymes similar to skeletal muscle (LDH<sub>4</sub>, LDH<sub>5</sub>; rabbit, horse, lamb, dog, humans) [29], in chicken and turkey, the patterns are opposite, with the prevalence of anodic isoenzymes similar to the aerobic heart muscle isoenzyme [27, 28]. The reason for these differences is not clear but it may suggest that the bird internal organs are geared to function better under aerobic metabolism.

Besides the serum and tissue LDH isoenzymes of adult birds, we also succeeded in separating lactate dehydrogenases originated from selected chicken embryo tissues using IEF technique in the pH range of 3–9 (Table 2).

An interesting pattern was found in the muscle of chicken embryo where all five isoforms were present (Table 2), while in adult animal tissue, only one, LDH<sub>5</sub> isoenzyme, was detected (Figure 2). This difference can be related to the developmental changes of LDH isoenzymes, similar to heart chicken embryonic lactate dehydrogenases. They were also relatively equally distributed on the electrophoreogram producing a pattern different from adult chicken heart where anodic LDH<sub>1</sub> isoform prevails over cathodic ones that are either absent or present only in a trace amount.

| Tissue          | Lactate dehydrogenase (%) |    |    |    |    |
|-----------------|---------------------------|----|----|----|----|
|                 | 1                         | 2  | 3  | 4  | 5  |
| Skeletal muscle | 18                        | 21 | 21 | 20 | 20 |
| Heart           | 12                        | 21 | 23 | 21 | 18 |

**Table 2.** Relative distribution (%) of lactate dehydrogenase isoenzymes in skeletal muscle and heart of chicken embryo (n = 6).

## 7. Conclusion

A suitable method for electrophoretic separation of bird lactate dehydrogenase isoenzymes is isoelectric focusing technique in a pH range of 3–9. It enabled to determine patterns and relative distribution (%) of LDH isoforms in bird sera and tissues (chicken adult and embryonic, as well as turkey). They were characterized by a prevalence of slow moving LDH<sub>5</sub> in sera in both species probably originated from breast muscle that distinguish them from mammalian

pattern where anodic LDH<sub>1</sub>-LDH<sub>3</sub> dominate over cathodic form/s. Mammalian tissues pattern of lactate dehydrogenase isoenzymes differs from species to species with the highest enzyme activity in the skeletal muscle followed by heart and liver. Chicken adult and embryonic lactate dehydrogenases differ each other especially in the pattern of breast muscle with all five isoenzymes being present in the tissue of embryonic origin.

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## Conflict of interest

The authors have declared no conflict of interest.

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