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Review

Methods for the separation of lactate dehydrogenases and clinical significance of the enzyme

Gerhard Kopperschläger*, Jürgen Kirchberger

Institute of Biochemistry, Medical Faculty, University of Leipzig, Liebigstrasse 16, D-04103 Leipzig, Germany

Abstract

Lactate dehydrogenase (LDH), an ubiquitous enzyme among vertebrates, invertebrates, plants and microbes was discovered in the early period of enzymology. The enzyme has been dissolved in several distinguishable molecular forms. In mammals, three types of subunits encoded by the genes *Ldh-A*, *Ldh-B* and *Ldh-C* give rise to a selected number of tetrameric isoenzymes. LDH-A_4, LDH-B_4 and the mixed hybrid forms of the A- and B-subunits are present in many tissues but with certain distribution patterns. LDH-C_4 is confined in mammals to testes and sperm. Numerous techniques have been employed to purify, characterize and separate the different forms of the enzyme. This report deals with the main protocols and procedures of purification of LDH and its isoenzymes including chromatographic and electrophoretic methods, partitioning in aqueous two-phase systems and precipitation approaches. In particular, affinity separation techniques based on natural and pseudo-biospecific ligands are described in detail. In addition, basic physico-chemical and kinetic properties of the enzyme from different sources are summarized. In a second part, the clinical significance of the determination of LDH in diverse body fluids in respect to the total activity and the isoenzyme distribution in different organs is discussed.

Keywords: Lactate dehydrogenase; Enzymes

Contents

1. Introduction 26
2. Enzyme properties 27
   2.1. Kinetics 27
      2.1.1. Substrate specificity 27
      2.1.2. pH- and temperature optimum 28
      2.1.3. Activation 28
      2.1.4. Inhibition 28
      2.1.5. Reaction mechanism 28
      2.1.6. Methods for the determination of the enzyme activity 29
   2.2. Molecular properties 29
3. Characterization of lactate dehydrogenase isoenzymes 29
4. Methods for the purification of lactate dehydrogenase and separation of its isoenzymes 30
   4.1. General aspects of purification 30
      4.1.1. Ion-exchange chromatography 30
      4.1.2. Affinity chromatography 30

*Corresponding author.

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4.1.3. Affinity precipitation ... 36
4.1.4. Affinity partitioning in aqueous two-phase systems ... 37
4.2. Isoenzyme separation in mammalian tissues ... 40
4.3. Purification of lactate dehydrogenase from microbial sources and plants ... 42
5. Clinical significance ... 42
5.1. Standard assay conditions ... 42
5.2. Isoenzyme detection in clinical practice ... 43
5.3. Abnormal isoenzyme pattern ... 44
5.4. Genetic deficiencies and variants ... 44
5.5. Enzyme activity and isoenzyme pattern in diseases ... 44
5.5.1. General aspects ... 44
5.5.2. Indications ... 45
6. The significance of lactate dehydrogenase as an auxiliary enzyme in enzymatic assays ... 46
7. Lactate dehydrogenase and cell injury ... 46
8. List of abbreviations ... 46
References ... 46

1. Introduction

Lactate dehydrogenase (EC 1.1.1.27), L-(+)-lactate:NAD$^+$ oxidoreductase (LDH) is an ubiquitous enzyme among all vertebrate organisms. It catalyzes the final reaction of glycolysis, the formation of L-(+)-lactic acid. LDH is also present in many invertebrates, plants and diverse microorganisms. In a few animal species and several microbes also, D-(−)-lactate is oxidized by another stereoselective LDH [EC 1.1.1.28; D-(−)-lactate:NAD$^+$ oxidoreductase]. Three types of LDH were found in yeast which are quite different from those mentioned above and which are considered as metal-containing flavoproteins. There are L-(+)-lactate:cytochrome *c* reductase or cytochrome b_2 (EC 1.1.2.3), D-(−)-lactate:cytochrome *c* reductase (EC 1.1.2.4) and D-(−)-lactate dehydrogenase (EC 1.1.2.8). All these enzyme species catalyze irreversibly the conversion of lactate to pyruvate.

LDH catalysis was discovered firstly in cell-free muscle extracts which were found to oxidize L-lactate to pyruvate [1–3]. The enzyme was finally purified by Straub in 1940 [4] while the first crystals were obtained by Kubovitz and Ott in 1943 [5].

LDH has received great attention because of its significant metabolic role. The widespread occurrence of this enzyme is due to its function in the glycolytic metabolism. It permits organisms to overcome a temporary oxygen debt in the form of accumulated L-lactate to be later discharged by the reoxidation reaction to pyruvate when oxygen becomes available.

Animal LDH was found to be a tetrameric molecule [6] which exists in different isoenzymes [7,8]. These forms are generated by association of two genetically distinct subunits to homo- and heterotetramers. The oligomeric enzyme can be dissociated by freezing and thawing, by high salt concentrations or by treatment with denaturating agents. Sequence similarities allows association of subunits from different species in vitro to form functional tetramers [9]. At least two gene duplication events have led to three genetically distinct subunits, LDH-A or M(muscle)-type, LDH-B or H(heart)-type and LDH-C or X-type in vertebrates and each species has diverged substantially in physico-chemical properties, biological function and developmental regulation [10]. LDH-A_4, LDH-B_4 and their three hybrid forms are present in all vertebrates and the characteristic tissue-specific expression of each has remained relatively unaltered throughout their evolution [11,12]. The relative abundance of the five isoenzymes in each cell type is entirely dependent upon the numbers of subunits of each types available to combine. These numbers reflect the relative activity of the corresponding genes *Ldh-A* and *Ldh-B*.

In reptiles, amphibians and fishes however, combinations of the subunits is frequently restricted so that in the extreme case only homotetramers are formed.

The isoenzyme LDH-C has a comparatively re-

stricted tissue distribution among vertebrates. It is expressed in all mammals (and in the pigeon) in primary spermatocytes and in those cells alone [13,14]. The gene appears totally inactive in females. Furthermore, a different LDH-C was found in a single family of columbic birds [15] and in the retina and liver tissues of teleost fishes [16,17].

It has been argued that the vertebrate *Ldh* loci arose by gene duplication from an ancestral locus giving rise to *Ldh-A* and *Ldh-B* and that one or more subsequent duplications generated *Ldh-C*. But this hypothesis has been refined and it is now believed that *Ldh-C* of fishes and birds are independent duplications of *Ldh-B* and the mammalian *Ldh-C* derives from *Ldh-A*, since the genes encoding LDH-A and LDH-C are syntonic on chromosome 11 in human and mice. Furthermore, the retina and liver specific LDH-C of the teleost fishes is immunologically more similar to LDH-B than to LDH-A [17].

The tissue distribution of LDH isoenzymes varies within the mammalian species. Originally, the LDH-A_4 which was designated according to its lowest electrophoretic mobility as LDH-5, was thought to be predominantly in skeletal muscle of all species whereas heart muscle seemed to express mainly the fast moving LDH-B_4 (LDH-1). On these findings, Kaplan and coworkers propounded the widely accepted theory concerning the differential physiological functions of the LDH isoenzymes, the socalled "aerobic–anaerobic theory" which postulates that the B-type LDH is suited to aerobic metabolism (e.g. heart muscle) while the A-type is more suited to tissues exposed to oxygen limitation, e.g., skeletal muscle [18]. However, this theory may be doubtful because several mammalian tissues show alterations of the "classical" distribution pattern. For example, human liver, an aerobic organ expresses the A-type of LDH while human erythrocytes, which perform exclusively glycolysis, synthesize predominantly the B-type. Furthermore, there are larger differences in the isoenzyme pattern of LDH within individual organs of different species. Liver, kidney medulla and erythrocytes of rat express mainly the A-type of LDH whereas the B-type was more significant in comparable organs of rabbit and bovine [19].

Structural properties and catalytic function of LDH from diverse biological sources have been investigated thoroughly. The particular interest for this enzyme is founded on its importance in carbohydrate metabolism, on its significance as a diagnostic parameter and on its value as a tool in the enzymatic analysis of other enzymes and of biological compounds.

2. Enzyme properties

2.1. Kinetics

LDH (EC. 1.1.1.27) catalyzes a straightforward equilibration of:

$$\text{L-lactate} + NAD^{+} \rightleftharpoons \text{pyruvate} + NADH + H^{+}$$

The formation of L-lactate is greatly favoured. The equilibrium constant, K, was determined at 25°C and calculated for pH 7.0 with [20]:

$$K = \frac{[\text{pyruvate}][NADH][H^{+}]}{[\text{L-lactate}][NAD^{+}]} = 2.8 \cdot 10^{-5}\ \text{mol l}^{-1}$$

The reaction between the oxidized substrate and the reduced coenzyme generates, as for most other dehydrogenases, large negative enthalpy and moderate positive entropy changes, respectively [21].

2.1.1. Substrate specificity

The enzyme is specific for L-(+)-lactate and does not react with D-(−)-lactate. LDH slowly oxidizes glyoxalate, glycerate and 3-halogen derivatives of L-lactate and 2,4-diketoacids. The reduction of 2-oxoacids decreased rapidly with increasing chain length of the acid [22]. The potential of the enzyme to reduce 2-oxobutyrate (2-oxobutyrate dehydrogenase (HBDH) activity, see Section 3 and 4) depends on the enzyme source.

LDH is specific for NADH. The phosphorylated derivative NADPH is almost not oxidized [23].

The K_m-values for L-lactate and NAD^+ of the vertebrate enzyme are significantly higher than those values for pyruvate and NADH, respectively. The higher affinity for NADH compared with NAD^+ was also corroborated by binding studies [23]. Representative K_m-values for various substrates depending on the enzyme source are summarized in [24,25].

2.1.2. pH- and temperature optimum

The pH-optimum of LDH is between pH 6 and pH 8 and depends in this range on the direction of the reaction and on the origin of the sample. For LDH from microbes, the pH optimum is shifted more to the acidic range (pH 4.5 to pH 6). The temperature optimum was determined for most of the enzymes between 39 and 45°C [24].

2.1.3. Activation

Mammalian LDH is activated by 2-amino-2-methyl-1-propanol, diethanolamine, fluoride and heparin [26]. Sodium sulphite protects the enzyme from and mercaptans reverse the inhibitory effects of thiol-attacking reagents [23,24]. LDH from various bacteria is activated by fructose 1,6-bisphosphate, fructose 2,6-bisphosphate and glucose 1,6-bisphosphate [27,28]. Bivalent cations like Mn^{2+}, Co^{2+}, Ca^{2+} stimulate some enzymes from microbial sources [24].

2.1.4. Inhibition

Generally, in mammals the enzyme is inhibited by pyruvate, high concentrations of lactate, NAD^+ and various carboxylic acids, particularly oxamate and oxalate. Oxamate was found as competitive inhibitor of pyruvate, conversely oxalate competes with lactate. Both inhibitors can form ternary complexes (1:1:1) with LDH–NAD^+ and LDH–NADH, respectively [29,30]. No inhibition was observed in the presence of cyanide and EDTA [23,26]. The enzyme is blocked by diverse metal cations like Ag^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and by thiol-attacking reagents [24,29].

The oxidation reaction (lactate→pyruvate) is inhibited by NAD^+-derivatives or substances which form complexes with enzyme bound NAD^+. The reduction reaction (pyruvate→lactate) is inhibited by NADH-derivatives which can be formed, in particular, during storage of phosphate buffered NADH solutions [23,26,31].

2.1.5. Reaction mechanism

In comparison with reactions of other dehydrogenases, the mechanism of LDH catalysis is rather simple. As a result of kinetic and equilibrium binding experiments, a compulsory order of binding, coenzyme first and substrate second was established. The rate limiting step in the oxidation of lactate at saturating concentrations of substrates is the rate of dissociation of NADH from the binary complex. In the reverse direction, the rate limiting step is either associated with the redox reaction or with the dissociation of the enzyme–NAD^+ complex [23]. As shown by fluorescence quenching, the enzyme during catalysis passes through eight different kinetically identifiable intermediates. The crystal structures of these distinct forms, i.e., the enzyme, the enzyme–NADH complex and the enzyme–NADH–substrate complex were analyzed [32]. A number of essential elements of the reaction mechanism for LDH are well understood [33]. The proposed situation of the active site of LDH is shown in Fig. 1. The imidazole ring of His_{195} interacts with substrates and performs two main functions: (1) proton donor/acceptor in the redox step and (2) proper substrate orientation for its interaction with the C_4 of NADH. When the substrates are bound, Asp_{168} interacts with His_{195} to stabilize the protonated form. Arg_{171} provides a strong two-point interaction with the substrate carboxylate. Arg_{109} serves to polarize the carbonyl bond of the ketoacid promoting the hydride transfer to the carbon and the proton transfer to the oxygen. Gln_{102} and Thr_{246} form the environment of the substrate chain and play a role in substrate discrimination. One of the prominent features of the coenzyme pocket in LDH is the hydrophobic environment of the nicotinamide ring represented by Ile_{250} [32,34]. This has been invoked to explain the tighter binding of the neutral NADH over the charged NAD^+.

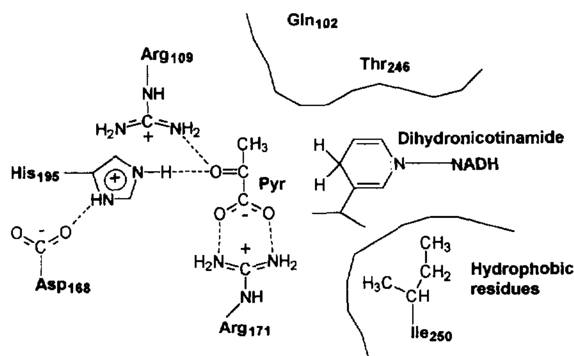

Fig. 1. Schematic summary of the reaction mechanism of lactate dehydrogenase showing the active site with bound subtrate and coenzyme and those amino acid residues which are important in this interaction (From Ref. [33] with permission).

The monomeric form of the enzyme is inactive [35]. Fluorescence and difference spectroscopy as well as ultracentrifugation have shown that the tetrameric form of vertebrate LDH has four independent non-interacting binding sites for NADH, NAD^+ and the substrates, respectively. Both dinucleotides compete for the same binding site. Lactate as well as pyruvate do not bind to the apoenzyme [23].

LDH from various *streptococci*, *bifidobacteria*, *lactobacilli*, *fermentative mycoplasmas*, *staphylococci*, *micrococci* and an *actinomycete* was found to be allosteric because fructose 1,6-bisphosphate acts as positive effector [36–38]. In some microbes, like *Streptococcus uberis* and *Bacillus stearothermophilus*, the enzyme is completely inactive if this effector is not present. In some cases, the association–dissociation equilibrium of LDH is governed by fructose 1,6-bisphosphate [27,39].

LDH from leaves of higher plants (lettuce) also shows allosteric properties. ATP was found as competitive inhibitor with respect to NADH. The existence of regulatory binding sites for the enzyme are discussed in [40]. Some of the plant species accept both NAD^+ and $NADP^+$ as coenzyme [41].

2.1.6. Methods for the determination of the enzyme activity

The most common procedure for the determination of LDH activity is the UV method using pyruvate/NADH or L-(+)-lactate/NAD^+ [26]. In addition, various colorimetric measurements utilizing different electron carriers and redox-indicators were developed [42].

2.2. Molecular properties

A comparison of the primary and tertiary structures of LDH from different sources shows that all of these enzymes belong to an evolutionary family with a common ancestor [10,43]. At present, complete amino acid sequences of LDH from various species are available in respective data banks [24]. Conspicuously, in the B-subunit of LDH, acidic amino acids and in the A-subunit, basic amino acids are dominating. Generally, there is a greater sequence similarity between genetically identical subunits of different species than between different subunits of the same species [44].

Although the enzyme contains more than two cysteine-residues, no disulphide bridges have been found. The Cys_{165} seems to be essential for catalysis but is not directly involved in the catalytic process [23,45]. The enzyme does not contain metal ions.

Three dimensional structures of LDH have been solved from five species, i.e., dogfish, porcine, mouse, *Bacillus stearothermophilus* and *Lactobacillus casei* and preliminary crystallographic data of the enzyme from *Bifidobacterium longum* and *Thermus caldophilus* are available [24,46].

As found in many other dehydrogenases, LDH (EC 1.1.1.27 and EC. 1.1.1.28) contains the highly conserved sequence Gly-X-Gly-X-X-Gly (where X can be any amino acid) in the middle of the $\beta\alpha\beta$-motif of the NAD^+-binding domain [47,48].

The quaternary structure varies depending on the source of the enzyme and in some cases, on the presence of structure-stabilizing effectors like the coenzyme or fructose 1,6-bisphosphate [39]. Both tetrameric and dimeric forms of the enzyme were found in vivo.

The molecular mass of the subunits of all isoenzymes including the D-(−)-specific LDH is relatively uniform and was found in the range of 34 000 to 40 000 [24].

Representative physico-chemical data like sedimentation and diffusion coefficient, partial specific volume, molecular mass, protein absorption coefficient, fluorescence characteristics, helix content, etc. of LDH from chicken, porcine, dogfish and bovine have been reviewed in Ref. [23].

3. Characterization of lactate dehydrogenase isoenzymes

In the range of pH 5.5–9.0, NADH is bound more weakly to LDH-A_4 than to LDH-B_4 [23,49]. Muscle LDH requires higher concentrations of pyruvate or lactate for attaining optimum than heart LDH. In fact, at optimum pyruvate concentration for muscle LDH, the heart enzyme is already inhibited [26]. The ability to use 2-oxobutyrate as substrate is a characteristic property of LDH-B_4 [50]. Urea (2 *M*) inhibits LDH-B_1A_3 and LDH-A_4 to a greater extent than LDH-B_4 and LDH-B_3A_1 [51]. Hybrid forms containing more B-type possess higher temperature

stability. Muscle LDH is cold labile. Heart LDH may be stored frozen [52].

Based on the unequal partition of basic and acidic amino acids in the B- and A-polypeptide chain of LDH, the isoelectric points of the individual isoenzymes are significantly different. For example, the isoelectric point of bovine heart LDH is 5.3 and that of rabbit muscle LDH is 8.5 [23]. The differences in the net charge have been used, more or less successfully, for the development of isoenzyme separation methods (see Section 4.2) and for the isoenzyme differentiation in clinical diagnosis (see Section 5).

LDH-C_4 is more sensitive to inhibitors like citrate, succinate, malate, aspartate and glutamate than LDH-B_4. Furthermore, this isoenzyme is less thermolabile. Oxamate and oxalate are competitive inhibitors. LDH-C_4 possesses a broad substrate specificity. It also catalyzes the reversible reduction of α-ketobutyrate, α-ketoglutarate, α-ketovalerate with NADH [9,53,54]. The urea inhibition is comparable to that of LDH-B_4 [54].

Complete sequences for mouse and rat LDH-C_4 have been published. Antibodies against the C-type of mammalian LDH do not crossreact with somatic LDH isoenzymes composed of A- and B-subunits, respectively [55]. Sequence analyses revealed that the mammalian LDH-C_4 is more different from either LDH-A_4 or LDH-B_4 than these two isoenzymes are from each other. The comparison of the structure of the three isoenzymes (A_4, B_4, C_4) showed some alterations in the three-dimensional structure [56]. The tertiary structure of LDH-C_4 showed the peculiarity that the flexible loop (amino acid residues 99–112) which covers the coenzyme after binding is already closed in the LDH-C_4 apoenzyme. This is in contrast to the other isoenzymes.

4. Methods for the purification of lactate dehydrogenase and separation of its isoenzymes

4.1. General aspects of purification

4.1.1. Ion-exchange chromatography

LDH is localized in the cytoplasm and can be liberated into solutions by mechanical or osmotic disruption of the cells. A common source for getting larger amounts of this enzyme is porcine or bovine heart which yields particularly the B_4 and the AB_3 isoenzymes. Since the first isolation of the enzyme by Straub in 1940 [4] applying Ca-phosphate adsorption, acetone and ammonium sulphate precipitation, the purification protocols has been improved continuously. The introduction of ion-exchange chromatography is considered as a milestone in the purification of the enzyme including the fractionation of the multiple enzyme forms. For example, by using QAE-cellulose as adsorbent and an elution buffer of pH 7.4, a stepwise fractionation of all five LDH isoenzymes can be performed by increasing the ionic strength [57]. Alternatively, the five isoenzymes have been eluted from DEAE-cellulose columns with increasing salt concentration (0–0.25 *M* NaCl) and decreasing pH (8.0–7.2) [58].

The HPLC version of protein ion-exchange chromatography has also been exploited for the purification of LDH. With this technique, not only much faster separation but also higher resolution was obtained. For example, LDH isoenzymes were successfully fractionated by anion-exchange chromatography on Synchropak AX-300 [59].

The evaluation of displacement chromatography for the purification of LDH from bovine heart under larger scale conditions has been studied in detail by Ghose and Mattiasson [60]. Using Tris Acryl DEAE M as a weak anion-exchanger and carboxymethyl-starch as displacer, this technique revealed distinct advantages over the conventional ion-exchange in terms of purity of the enzyme and the overall yield.

4.1.2. Affinity chromatography

The development of this separation technology is based on the principle of biorecognition and has provided specific methods for the purification of diverse proteins. Affinity chromatography is one of the oldest approaches to applying of this principle. Basically, the method includes the following steps: (1) binding of proteins to the immobilized ligand; (2) removal of unbound proteins by washing the matrix; (3) desorption of specifically bound proteins by competing ligands, increasing ionic strength or changing the pH of the elution buffer.

Nucleotide ligands. Two "general ligands", i.e., AMP and NAD^+, were introduced by Mosbach and

his group for the separation of dehydrogenases including LDH by forming binary complexes of the enzymes with the immobilized ligands [61].

Studies on the spacer length between the ligand and the matrix, which is frequently important for optimal enzyme binding, were performed by Lowe et al. [62]. For the binding of LDH, an extension length of 0.7–1.0 nm was found optimal and this was achieved by coupling the nucleotide via a 6-aminohexanoyl-group to the Sepharose matrix [61,62].

Alternatively, the pyruvate analogue oxamate, (see Section 2.1), has also been coupled to Sepharose resulting in a similar binding of LDH as in the case of NAD^+ [63].

The bound LDH can be dissolved from the column by addition of NADH (0.1–1 m*M*) or NAD^+ but the latter one is less effective than NADH [61]. However, the effectiveness of NAD^+ can be drastically enhanced when the NAD^+-containing buffer is completed with sulphite, lactate, pyruvate or oxamate which give rise to form strong ternary complexes with the enzyme [7,61,64].

Separation of various dehydrogenases can be achieved by choosing the correct nucleotide and concentration to desorb the enzymes specifically from columns which were loaded with a rough tissue extract. For example, glyceraldehyde-3-phosphate dehydrogenase and LDH have been separated in a column of N^6-(6-aminohexyl)–NAD^+–Sepharose by eluting the former enzyme with 0.15 m*M* NAD^+ and the latter one with 0.15 m*M* NADH [61].

The different affinity of LDH isoenzymes for the nucleotide substrate can also be exploited for the separation of multiple forms. The order of the elution of the five isoenzymes correlates with the relative affinity of NADH in solution [65] (see Fig. 8 in Section 4.2).

Although affinity chromatography with immobilized natural ligands exhibits a fascinating approach, as well as benefits in terms of selectivity, recovery and effectiveness, its large-scale application however, is restricted by the high costs of preparing sufficient amounts of labeled matrices and of the chemical instability of most of the ligands.

Dye ligands. The discovery of reactive textile dyes exhibiting a "pseudo-biospecific" affinity to many enzymes and other proteins led to the development of a special branch of affinity separation technique. A large number of this class of dyes mimic, more or less accurately, structures of natural ligands and are able to bind to the natural counterpart at the protein. Fortunately, these dyes are available as low-cost chemicals in large amounts. They are reactive and therefore easy to couple to diverse matrices under mild chemical conditions. Many of them show a certain biospecificity particularly to dehydrogenases, kinases and other nucleotide-dependent enzymes [66].

The affinity of LDH to Blue Dextran, a conjugate of dextran and Cibacron Blue F3G-A, was firstly reported by Stellwagen et al. [67] who could demonstrate also the reversibility of binding by eluting the enzyme from a Blue Dextran–Sepharose column with NADH.

In Fig. 2, the structures of three types of dyes exhibiting affinity to LDH and which belong to different classes of reactive dyes are depicted. The extent of binding of the enzyme to the immobilized dye and the recovery of desorbed LDH depends on several factors. These are: (1) structural properties of the dye; (2) degree of matrix substitution; (3) chemistry of coupling the dye to the matrix; (4) property of the matrix.

One example is presented in Table 1 which shows the dependence of the binding capacity, recovery and purification factor on diverse dyes tested [68].

For improving binding capacity and recovery of LDH, it seems to be advantageous to couple the biomimetic dye via a spacer to the support. Results of a thorough study applying bead cellulose are shown in Table 2, which clearly indicate that an extension arm of a certain length optimizes binding and eluting of the enzyme. Looking in more detail at the results of Table 2, differences between Procion Green H-4G and Procion Scarlet MX-G in respect to the conditions of eluting the LDH become evident. Although 1 *M* KCl is able to desorb most of the enzyme from the affinity matrix of Procion Green H-4G independent of the spacer length, the enzyme was almost completely retained on the Procion Scarlet MX-G and Procion Orange MX-G matrix under the same conditions. Decreasing or increasing the ionic strength of the elution buffer did not essentially improve the yield of the enzyme. However, desorption of the enzyme occurred on applica-

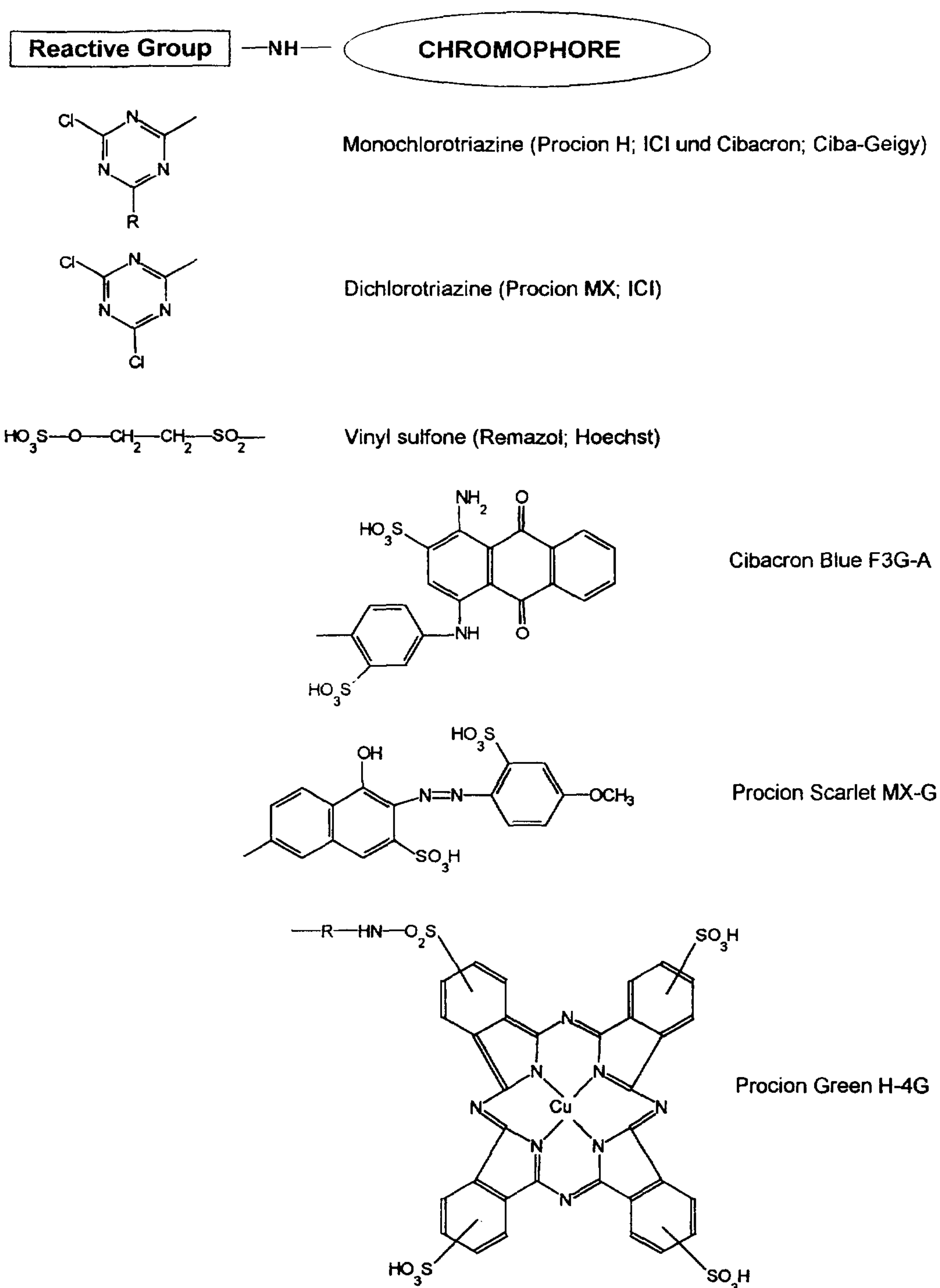

Fig. 2. Schematic structure of reactive dyes used as biomimetic ligands in the recognition of lactate dehydrogenase

Table 1
Binding capacities of dye derivatives of bead cellulose and recovery of eluted lactate dehydrogenase (From Ref. [68] with permission)

Dye	Degree of dye substitution [mg dye/g cellulose (wet weight)]	Binding capacity of the dye-bead cellulose for LDH [units/g cellulose (wet weight)]	LDH activity eluted in the main fraction (%)	Purification factor (*n*-fold)
Cibracron Blue F3G-A	2.0	200–245	55–65	3–4
Procion dyes				
Red HE-3B	2.0	10–30	70	
Red HE-7B	5.7	190–270	75–90	n.d.
Navy H-ER	3.7	140–200	80–90	4–5
Brown HE-G	n.d.	70–150	50–75	5–6
Yellow HE-4R	3.2	100–250	70	3
Scarlet MX-G	3.8	No binding	–	3–4
Orange MX-G	1.2	No binding	–	–
Green H-4G	2.0	No binding	–	–
Yellow HE-3G	1.3	No binding	–	–
Remazol dyes				
Brilliant Blue R	20.0	100–250	80–90	3–5
Brilliant Red 5 BN	n.d.	100–300	70–90	3–5

The binding capacity of dye–bead cellulose was determined using bovine heart lactate dehydrogenase prepurified by DEAE–Sephadex chromatography (specific activity 30–70 U/mg). Columns (10cm × 1cm) containing 5 g of the respective dye–cellulose were equilibrated with 20 m*M* potassium phosphate, pH 7.0, 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol. The columns were loaded with an excess of LDH (about 400 units/g cellulose) at 10°C. The amount of activity adsorbed (defined as 100%) was calculated from the difference between the total activity of LDH loaded onto the column and the unbound one determined in the breakthrough fraction and the wash pool. The purification factor was calculated from the increase in the specific activity of the enzyme.
n.d. = not determined.

tion of a mixture of NAD^+–sulphite and a higher recovery from the column was also found with growing spacer length of the ligand. It is therefore probable that in some cases the spacer arm gives rise to additional hydrophobic interactions with LDH which are strengthened by increasing salt. That means that the enzyme can be eluted from the column in the presence of competing effectors only at low ionic strength.

Based on these findings the enzyme has been purified to homogeneity from a crude extract in one step (Table 3) [68].

Improved elution of LDH from Cibacron Blue F3G-A–Sepharose and Procion Scarlet H-2G–Sepharose was found when the affinity matrix was shielded with poly(N-vinylpyrrolidone) [69]. This type of modification is considered to prevent the matrix from binding foreign proteins and from unspecific binding of nucleotide-dependent enzymes, while not seriously impairing the specific adsorption of these enzymes to the affinity matrix. Furthermore, poly(N-vinylpyrrolidone and poly(N-vinylcaprolactam) themselves are more efficient eluting agents of porcine muscle LDH bound to Cibacron F3G-A–Sepharose than NADH and oxamate or high salt concentrations [70].

In addition, a temperature-induced displacement of LDH from Cibacron F3G-A–Sepharose using an immobilized poly(N-vinylcaprolactam) displacer was demonstrated [71]. The globule–coil transition of the polymer, exhibiting a cloud point of 38°C, was exploited for the displacement of bound LDH. The enzyme was bound to the polymer-shielded column at 40°C. At this temperature, the enzyme could not be eluted from the column with 0.1 *M* KCl but unbound proteins were washed out. The decrease in temperature to 23°C resulted in LDH elution with 0.1 *M* KCl. Starting from crude porcine muscle extract the recovery of the enzyme was about 90% and the purification factor was 17.

The choice of the matrix for coupling the bio-specific ligand is often important, not only for

Table 2
Binding capacity and eluation behaviour of dye–(spacer)–bead cellulose to lactate dehydrogenase from bovine heart (From Ref. [68] with permission)

Adsorbent (Dye–C_n–BC)[a]	Binding capacity (units/g wet adsorbent	Percentage of eluted enzyme activity of LDH in presence of	
		1 *M* KCl	0.05 m*M* NAD^+–1 m*M* sulphite
Procion Red HE-3B			
–C_6–BC	30–60	n.d.	29
Cibacron Blue F3G-A			
–C_6–BC	150	n.d.	47
Procion Green H-4G			
–C_2–BC	450–620	84	35
–C_4–BC	500–640	92	51
–C_6–BC	500–680	75	84
Procion Scarlet MX-G			
–C_2–BC	500–650	5–10	31
–C_2–BC	200–300**	n.d.	30
–C_6–BC	500–770	5–10	79
Procion Orange MX-G			
–C_6–BC	200**	2–5	87
Procion Yellow HE-3G			
–C_6–BC	520	n.d.	30

[a] Abbreviation: Dye–C_n–BC = Dye–NH$(CH_2)_n$NH-CO-O–bead cellulose. Lactate hydrogenase with two different specific activities of 70 or 30 units/mg (marked by **) was applied. The percentage of the eluted activity is related to the adsorbed activity (defined as 100%). The latter was calculated from the difference between the total activity of LDH loaded onto the column and the portion unbound after washing the column with 20 m*M* potassium phosphate buffer, pH 7.0, 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol at 10°C.
n.d. = not determined.

proceeding the coupling but also for the quality of the final product since unspecific binding of proteins can impair the specific activity. For affinity chromatography of LDH the following supports have been used: Sepharose [67,72]; bead cellulose [68,73]; hydroxyethylcellulose [74]; Sephacryl S-200 [75]; Eudragit [76]; agarose beads [77].

The introduction of high-performance liquid affinity chromatography (HPLAC) gave rise to the development of a new class of dye-matrices based on spherical or microparticulate porous silica (Spherisorb, Lichrosorb Si 60) which were exclusively applied in the HPLC mode [78,79].

LDH was considered to be a suitable model

Table 3
Purification of lactate dehydrogenase from bovine heart by applying Procion Scarlet MX-G-(diaminohexyl)–bead cellulose (From Ref. [68] with permission)

Step	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor (n-fold)
Extract	47	1700	5.8	100	1
Dye–cellulose	14	891	200	52	34
Ultrafiltration	1.5	750	210	44	36

Experimental conditions: extract obtained from 30 g of minced bovine heart muscle was centrifuged (9000 *g*, 30 min) and the supernatant was loaded onto a column (2×20 cm) packed with 14 g of dye–cellulose. After washing with 20 m*M* potassium phosphate buffer, pH 7.0, 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol (equilibration buffer) containing additionally 1 *M* KCl and equilibration buffer without KCl, the enzyme was desorbed by NAD^+–sulphite (0.05 m*M*:1 m*M*) in equilibration buffer at 10°C.

protein for evaluating the spacer-mediated and direct-linked triazine dye HPLAC adsorbents. The specificity of adsorption of LDH was much weaker when applying the spacer-mediated affinity matrix in low ionic strength buffers, suggesting a significant contribution from hydrophobic forces in the interaction of proteins with the immobilized dye. This hydrophobic interaction is likely to be a consequence of the comparatively long polymethylene spacer molecule interposed between the silica surface and the dye (Cibacron Blue F3G-A). In this case, non-specific interactions can be circumvented by coupling the dye directly to the glycol-silylated silica. This coupling has been successfully performed with the more reactive dichlorotriazine dye, Procion Blue MX-R [79]. Fig. 3 depicts an example of HPLAC of rabbit muscle extract in which 80% of LDH was recovered by desorption of the enzyme with 2 mM NADH [80]. By scaling up this protocol to about 60 000 units (1.8 g protein), an overall yield of 47% and a specific activity of about 300 units/mg protein was obtained [81].

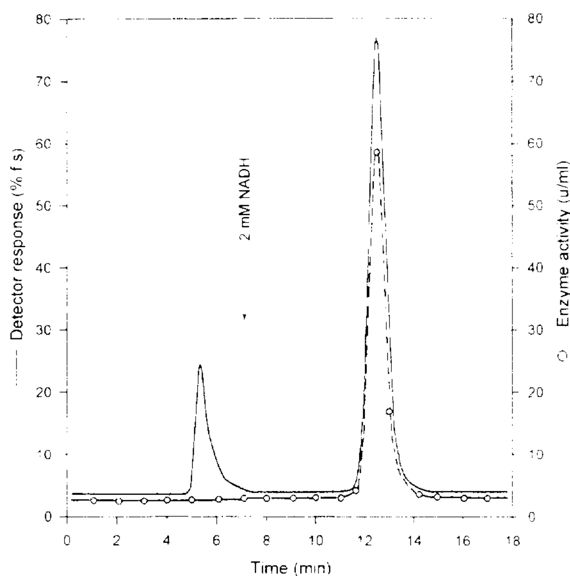

Fig. 3. Purification of rabbit muscle lactate dehydrogenase on a Procion Blue MX-R–silica column. Crude enzyme extract in 10 mM Hepes, pH 7.0 (1 ml, 13.9 mg, 79 U/mg) applied at time zero; temperature: 20–22°C; flow-rate: 10 ml/min; pressure: 3.5 MPa (500 p.s.i.); detector: 280 nm; eluent: 2 mM NADH (2 ml) in 10 mM Hepes, pH 7.0 as indicated by the arrow. Hepes = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. (From Ref. [80] with permission.)

Metal–chelate ligands. Immobilized metal–chelates have been found as another group of "general ligands" capable of binding various proteins [82]. Studies on the mechanism of adsorption on immobilized transition metals, such as Cu^{2+}, Ni^{2+} and Zn^{2+}, indicate that histidyl groups are the dominant electron donors. The numbers and sometimes the distribution of the histidyl groups dictate almost exclusively their retention behaviour on these immobilized metals. The latter proceeds when iminodiacetate was coupled covalently to agarose (IDA–agarose) which complexes diverse transition metals. Recently, it was shown that LDH from chicken muscle binds strongly to Ni^{2+} and Fe^{3+} [83,84]. With regards to the immobilized metal ion affinity chromatography (IMAC), the A-type of LDH has a particular amino acid sequence: the residues no. 5, 8, 11, 15, 17, and 19 are histidyl residues [85]. The occurrence of six histidines in a stretch of 20 residues is obviously the chemical basis for the strong binding of LDH-A_4. The B-subunit, not containing a single residue in the same region, was not bound. Therefore, IMAC should be able to separate both forms of enzymes which has been demonstrated so far.

A procedure describing the purification of LDH from chicken muscle to homogeneity in one step by applying a Ni^{2+}–IDA–Sepharose column has been reported [84].

Colchicine ligand. LDH from brain and other mammalian tissues was found to bind to immobilized colchicine at high concentration of NaCl [86]. This unexpected behaviour has been exploited to purify the enzyme by affinity chromatography in one step. A crude cytosolic extract was loaded onto a column containing colchicine–CH–Sepharose which had been equilibrated before with potassium phosphate buffer containing 0.5 M NaCl. After washing the column with the same buffer, the enzyme was desorbed with 1 mM NADH-equilibrating buffer and it appeared in a sharp peak. The yield was about 80%, the enrichment was 300-fold. For example, starting from 1 kg of bovine brain and using a

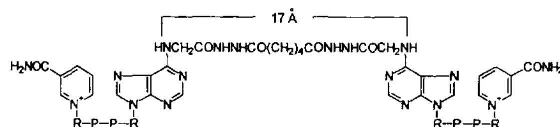

Fig. 4. Structure of bis-NAD$^+$ = N_2,N_2'-adipodihydrazido-bis-(N^6-carbonylmethyl-NAD$^+$) (From Ref. [87] with permission).

column (5 cm I.D.×4 cm) about 70 mg of pure enzyme was recovered.

4.1.3. Affinity precipitation

The technique of affinity precipitation combines a classic step of protein enrichment with the principle of biorecognition. The use of this approach has been demonstrated with a bifunctional NAD$^+$-derivative which was introduced by Larsson and Mosbach [87]. Bis-NAD$^+$ (N_2,N_2'-adipodihydrazido-bis-[N^6-carbonylmethyl-NAD$^+$]) (Fig. 4) was synthesized from adipic acid dihydrochloride and N^6-carboxymethyl-NAD$^+$. When this compound was mixed with certain NAD$^+$-dependent dehydrogenases in an equimolar ratio of NAD$^+$ equivalents to the NAD$^+$-binding sites, an almost quantitative precipitation of the enzymes resulted. For redissolving, the precipitate was treated with a competitive ligand such as NADH in sufficient concentrations.

The tetrameric structure of LDH is suited to form large aggregates with bis-NAD$^+$ because each subunit is potentially able to complex with half of the bis-dinucleotide. Finally, the formed aggregate becomes insoluble and precipitated out. As shown first by Flygare et al. [88], LDH could be precipitated directly from a crude extract of bovine heart. The purification protocol is presented in Table 4 and resulted in an overall yield of 80% and a purity of 95%. In comparison with other affinity separation techniques, the equipment demand for affinity precipitation is low, the operation speed high and the specificity sufficient.

However, the versatility of this approach for large scale use is constrained by the expense and lability of the NAD$^+$-derivative. In order to overcome the high costs and low stability of bis-NAD$^+$, the biomimetic dye Cibacron Blue F3G-A has been used to synthesize a bis-dye molecule for LDH precipitation [89]. This was obtained by coupling an aminohexyl-derivative of the dye with a non-modified dye via the carbodiimide condensing procedure. Applying a mixture of three proteins (bovine albumin, chymosin and LDH), the dehydrogenase was precipitated to 90% recovery and was redissolved by adding 0.1 m*M* NAD$^+$ into the buffer. On the other hand, complications encountered using Cibacron Blue F3G-A as a ligand for affinity precipitation of LDH were reported by Morris and Fisher [90]. In this attempt, the bis-dye was synthesized by coupling the monomer via the reactive chlorine group of the triazine ring with a diamine. The bifunctional molecule was obviously unable to form precipitates with LDH. One explanation for this behaviour could be that the dye itself gives rise to stacking interaction which prevents extensive cross-linking of LDH.

A rather selective precipitation of rabbit muscle LDH was demonstrated by using a Procion Blue analogue, i.e. the methoxylated *p*-sulfonate isomer of Procion Blue H-B [91]. Procion Blue H-B is closely related to Cibacron Blue F3G-A, the latter is a mixture of meta- and para-isomers. In this dye, the reactive chlorine group of the triazine ring was substituted by an methoxy group. Although this type of dye does not exhibit any bifunctional properties, a complete precipitation of LDH could be obtained if the molar ratio of the dye/LDH (subunit) was

Table 4
Purification of lactate dehydrogenase from bovine heart (From Ref. [88] with permission)

Stage	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (*n*-fold)	Recovery (%)
Crude extract	200	6500	18 700	3	1	100
Affinity precipitate	–	141	17 000	121	40	91
Dissolved precipitate after dialysis	10	117	16 800	144	48	90
Enzyme crystals in $(NH_4)_2SO_4$	10	100	14 950	150	50	80

adjusted to approximately two. Centrifugation of the precipitate and redissolving with NADH yielded a homogeneous enzyme as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The recovery of the enzyme activity was about 97% giving a six-fold overall purification as checked by the increase of the specific activity of the crude extract to 336 U/mg. The mechanism of precipitation described above seems to be different from those dye-promoted precipitations of proteins at low pH in which the negative charged dye molecules adsorb to an oppositely charged protein surface resulting in neutralized complexes which subsequently form insoluble aggregates [92]. The precipitation of LDH with methoxylated Procion Blue H-B occurs under pH conditions where the enzyme is neutral or negatively charged. Precipitation is observed exclusively with the para-isomer of the methoxylated dye. This behaviour and the competitive effect of NADH suggest a rather specific interaction between the dye and the enzyme.

A special mode of affinity precipitation of LDH, which was called affinity thermoprecipitation, has been developed by Guoqiang et al. [93]. In this approach Eudragit, a copolymer of methacrylic acid and methylmetacrylate was used as a carrier for the covalent binding of Cibacron Blue F3G-A. The solubility properties of the polymer are dependent on the medium pH being soluble above pH 5.8 and insoluble at pH 4.8 and below. In addition, the polymer could be precipitated with Ca^{2+} of high concentration and this effect was enhanced at higher temperatures. After coupling the dye to the matrix, the efficiency of precipitation at low pH could be enhanced by moderate increase of the temperature in the presence of low concentrations of Ca^{2+}.

Affinity precipitation of LDH has been also successfully elaborated with commercial soluble Blue Dextran, i.e., Cibacron Blue F3G-A bound to Dextran 500 [94]. Since the enzyme and the dye–polymer are polyvalent, there is a risk of spontaneous formation of affinity complexes large enough to induce precipitation. This happened in few cases, but only to a very low degree. After the complexes were formed, they were precipitated by addition of the lectin, concanavalin A ConA, which binds the dextran residues and induces precipitation. The efficiency of precipitating the enzyme was 85%. Dissociation of the bound LDH was carried out using 1.5 *M* KCl. The ConA-Blue–Dextran complexes were dissociated using glucose as competing ligand.

4.1.4. Affinity partitioning in aqueous two-phase systems

Aqueous two-phase systems have been used for more than three decades in biochemical research as tools for fractionation of proteins, nucleic acids, cell particles and cells. The interest in applying this principle for large-scale processes in biotechnology has grown strongly during the last decades.

Two-phase systems form spontaneously upon mixing aqueous solutions of two hydrophilic polymers when certain threshold concentrations are exceeded [95]. The partitioning of proteins depends on the chemical nature of the phase-forming polymers, their molecular weights and their concentrations. Ionic strength and pH of the medium also affect the distribution of proteins. Furthermore, intrinsic properties of the proteins, such as size, conformation, charge and hydrophobicity are important factors for their partitioning behaviour. The partition coefficient, defined as the ratio of the protein concentration in the upper and lower phases, is a characteristic property and can be viewed as the sum of individual terms;

$$\ln K = \ln K_{\text{charge}} + \ln K_{\text{hydrophobic}} + \ln K_{\text{hydrophilic}} + \ln K_{\text{conformation}} + \ln K_{\text{ligand}} + \ldots$$

Many proteins have unique interactions with ligands that offer the possibility of altering partitioning in a rational way and achieving selectivity. Such an approach, called affinity partitioning, combines the partitioning behaviour of macromolecules in aqueous two-phase systems with the principle of biorecognition. The biospecific ligands are covalently coupled to one of the phase-forming polymers, most often to poly(ethylene glycol) (PEG). Thus, in a PEG/dextran system the ligand–PEG will accumulate in the upper phase. If a protein of interest exhibiting affinity to a certain ligand is preferentially located in the lower phase, the ligand–PEG–protein complex formation causes a shift of the corresponding protein into the upper phase. This can be followed by measuring the change in the partition coefficient and the extent can be expressed by the

term Δlog K, i.e. the difference of the logarithms of the partition coefficients in presence and absence of the ligand–PEG.

Although natural cofactors of enzymes are suited to act biospecifically in affinity partitioning, their practical use is still not widespread because the procedures for coupling the ligand to the polymer is often laborious and the high cost of the ligand restrict a large-scale application. The discovery of biomimetic dyes has advanced affinity partitioning in the past decade, as well as its use for analytical studies and for purification of proteins [96].

Affinity partitioning as an analytical approach. Dye ligand affinity partitioning has been elaborated as a sensitive method for studying enzyme–dye interactions. As a rule, the dye ligand is coupled to PEG because of the ease of steering the partition of proteins, in the absence of the ligand–PEG, into the opposite dextran-rich phase. The effect of affinity partitioning can be quantified by determining the Δlog K-value. This measure, when plotted against the concentration of the dye–PEG, mostly followed a saturation function. Therefore, two parameters, Δlog K_{max} (maximum extraction power) and the half saturation point (0.5Δlog K_{max}), of the curve can be calculated. The value of Δlog K_{max} is related to the number of binding sites [96] whereas 0.5Δlog K_{max} is valid as a relative measure of the affinity of the ligand to the enzyme if identical conditions are compared. LDH has been found an excellent example to study the efficiency of affinity partitioning for analytical purposes. The enzyme from rabbit muscle was partitioned in aqueous two-phase systems composed of PEG/dextran containing Procion Red HE-3B and structurally related dyes coupled to PEG [97]. The structures of the dyes used are drawn in Fig. 5. The increase of the term Δlog K with increasing ligand–PEG concentration is shown in Fig. 6, providing similar hyperbolic curves with the exception of Procion H-3B and Procion HE-3B(M1). The calculated Δlog K_{max}-values and the half saturation points with this dye–PEG's and including two other well known biomimetic dye ligands are summarized in Table 5. No large differences in the Δlog K_{max}-values for all dyes tested have been found, but the half saturation points 0.5Δlog K_{max} were significantly different for the individual dyes. The

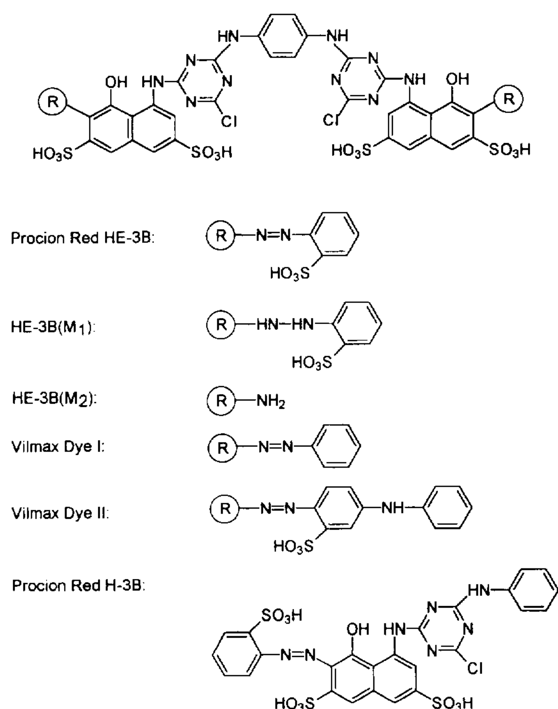

Fig. 5. Structures of Procion Red HE-3B derivatives used for affinity partitioning of lactate dehydrogenase from rabbit muscle. (From Ref. [97] with permission.)

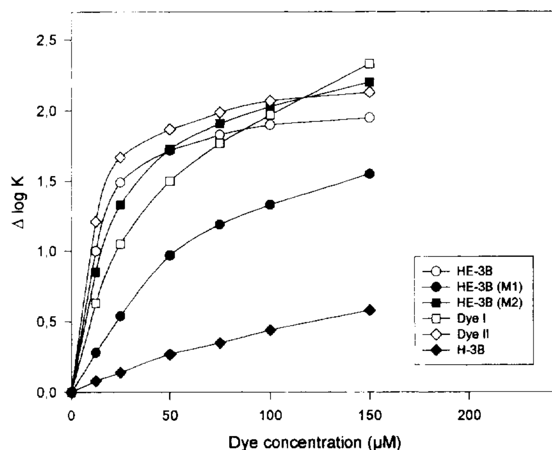

Fig. 6. Affinity partitioning of lactate dehydrogenase from rabbit muscle as function of the concentration of various dye–PEG derivatives. The systems (4 g) contained 9% (w/w) Dextran 500, 6% (w/w) PEG 6000 (partially replaced by dye-liganded PEG), 50 mM sodium phosphate buffer, pH 7.5 and 10 units of the enzyme. The systems were equilibrated at 25°C. The structures of the dye–ligands used are shown in Fig. 5. (From Ref. [97] with permission.)

Table 5
Affinity partitioning of lactate dehydrogenase of rabbit muscle expressed by the maximal extraction power ($\Delta\log K_{max}$) and the relative affinity ($0.5\Delta\log K_{max}$) using different dye–PEG derivatives (From Ref. [97] with permission)

Dye–PEG	$\Delta\log K_{max}$	Dye–PEG (μM) yielding $0.5\Delta\log K_{max}$
Procion Red HE-3B	2.08	9.5
Red HE-3B(M1)	2.27	67.1
Red HE-3B(M2)	2.53	23.5
Vilmax Dye I	2.63	36.4
Vilmax Dye II	2.32	10.4
Cibacron Blue F3G-A	2.56	44.4
Procion Yellow HE-3G	2.63	7.2

The systems (4 g) contained 9% (w/w) Dextran 500, 6% (w/w) PEG 6000 (partially replaced by dye–PEG), 50 m*M* sodium phosphate buffer, pH 7.5 and 10 units of the enzyme. The systems were equilibrated at 25°C. The structures of the dye–ligands used are shown in Fig. 5. The data were calculated from double reciprocal plots of $\Delta\log K$ as a function of the dye–PEG concentration (see Fig. 6). The regression coefficients were between 0.99 and 1.00.

lowest value of this parameter means the highest affinity of the dye and vice versa. Interestingly, Procion Red HE-3B and Procion Yellow HE-3G possess remarkable higher affinity to LDH compared to the most often applied dye, Cibacron Blue F3G-A.

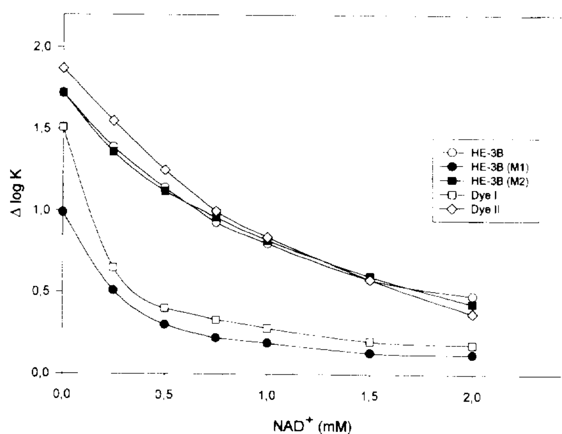

Fig. 7. Affinity partitioning of lactate dehydrogenase from rabbit muscle as function of the amount of NAD^+ added to the system. The systems (4 g) contained 9% (w/w) Dextran 500, 6% (w/w) PEG 6000 (including 50 μ*M* dye–PEG), 50 m*M* sodium phosphate buffer, pH 7.5 and 10 units of the enzyme. The systems were equilibrated at 25°C. The structures of the dye–ligands used are shown in Fig. 5. (From Ref. [97] with permission.)

In order to analyze the LDH–dye interaction with respect to its specificity to the nucleotide-binding domain, the technique of affinity partitioning is suited for measuring competing effectors. One example is presented in Fig. 7 in which the decrease of $\Delta\log K$ by increasing concentration of NAD^+ is demonstrated. The dinucleotide competes most effectively with Procion Red HE-3B(M1) and Vilmax Dye I in comparison to the other dyes used. The differences become dominant particularly at low concentrations of NAD^+.

Results of such studys provided contributions to finding suitable dye–ligands for optimal binding and competing effectors for sufficient desorption of the enzyme. In addition, the structural requirement of the dye–enzyme interaction can be estimated. However, the maximum extraction power $\Delta\log K_{max}$ cannot be used in a linear relation to predict the efficiency in chromatographic systems. Although a low value of $\Delta\log K_{max}$ is related to the unsuitability of the ligand for affinity chromatography, a higher value does not reflect per se its usefulness to function as ligand in affinity chromatography [68].

Purification of LDH by affinity partitioning. A number of commercial reactive dyes was screened to optimize conditions for LDH purification by affinity partitioning [98,99]. Based on this study, Procion Yellow HE-3G coupled to PEG 8000 has been selected as ligand. A thorough examination of all parameters influencing the partition of LDH and the bulk proteins of a crude muscle extract led to a protocol which allowed the purification of the enzyme in a single step (Table 6) [100].

Starting from 1 kg of a two-phase system containing 100 g porcine muscle extract, the first affinity partitioning step yielded an upper phase containing over 90% of the enzyme, which appeared 10-fold enriched. After separating the upper phase and washing twice with a fresh lower phase, the purification improved to homogeneity of the enzyme. Finally, the enzyme was desorbed from the dye–PEG by addition of phosphate buffer of high concentration which gave rise to form a new PEG/salt system. LDH was recovered in the lower salt-rich phase with 92% yield.

Table 6
Extraction of lactate dehydrogenase from porcine muscle extract (From Ref. [100] with permission)

Partition step[a]	Phase	Specific activity (U/mg protein)	Purification factor (*n*-fold)	Recovery (%)
Extract	–	23.6	1.0	100
Affinity partition	Upper	246	10.4	92
Wash step 1	Upper	428	18.1	86
Wash step 2	Upper	485	20.6	79

[a] System composition: 10% dextran, 7.0% PEG, 0.07% Procion Yellow–PEG, 50 m*M* sodium phosphate buffer, pH 7.9 and 25% muscle extract. Temperature: 0°C. After the affinity partitioning step, the upper phase of the system was washed twice with the same volume of pure lower phase.

This purification protocol was successfully scaled-up by using Aquaphase PPT, a low-cost polymer, instead of dextran and PEG. Applying a small centrifugal separator to enhance phase separation 3–5 kg crude extract/h could be processed in a 30-l two-phase system [101]. A specific approach has been elaborated in which affinity precipitation was combined with aqueous two-phase extraction [102]. Eudragit S 100 was used as dye–ligand carrier which partitioned in soluble form almost completely in the upper PEG-containing phase. Whereas the bulk proteins were concentrated in the lower, dextran-containing phase, the upper phase extracted those proteins exhibiting affinity to the dye Cibacron Blue 3GA. After phase separation, the Eudragit–dye–target protein complex was precipitated out from the top phase by lowering the pH to 5.1 according to the property of this polymer. LDH was recovered by treatment of the precipitate with 0.5 *M* NaCl with a yield of 54% and a specific activity of 245 U/mg.

4.2. Isoenzyme separation in mammalian tissues

For the separation of LDH isoenzymes, several methods like electrophoresis, anion-exchange chromatography, affinity precipitation [103], affinity partitioning and affinity chromatography using various ligands have been elaborated.

Separation by electrophoresis and by ion-exchange chromatography are due to differences in the isoelectric points of both types of subunits (see Section 3). Consequently, the hybrid forms of LDH are charged corresponding to the ratio of the A- and B-type of the subunits. Polyacrylamide in concentrations of 7–10% of the monomer and an alkaline buffer of pH 8.0–8.5 are preferentially used for separation of the isoenzymes.

As a rule, an anion-exchanger adsorbs LDH-B_4 much more strongly than the respective hybrid forms [58]. The corresponding LDH-A_4 is not bound under conditions at pH>8.0 and appears in the break-through [104].

Affinity chromatography was found as another approach to separate LDH isoenzymes. Using N^6-(6-aminohexyl)–AMP–Sepharose, the different forms of LDH could be eluted with an increasing gradient

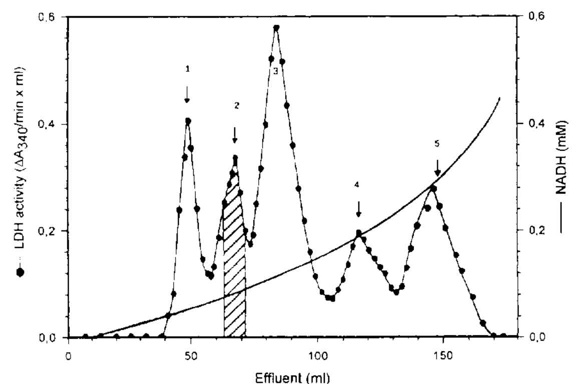

Fig. 8. Elution of lactate dehydrogenase isoenzyme mixture of bovine heart and muscle using a concave gradient of NADH. 0.2 mg of protein in 0.2 ml sodium phosphate buffer, pH 7.0, 1 m*M* 2-mercaptoethanol and 1 *M* NaCl were applied to an AMP-column (140×6 mm containing 2.5 g of wet gel), equilibrated with 0.1 *M* sodium phosphate buffer, pH 7.5. After washing the column with this buffer (10 ml), the isoenzymes were eluted with a concave gradient of 0–0.5 m*M* NADH in the same buffer containing 1 m*M* 2-mercaptoethanol. 1 ml fractions were collected at a rate of 3.4 ml/h. The arrows indicate the partition of the isoenzymes in the fractions. 1, LDH-B_4; 2, LDH-B_3A_1; 3, LDH-B_2A_2; 4, LDH-B_1A_3; 5, LDH-A_4. (From Ref. [88] with permission.)

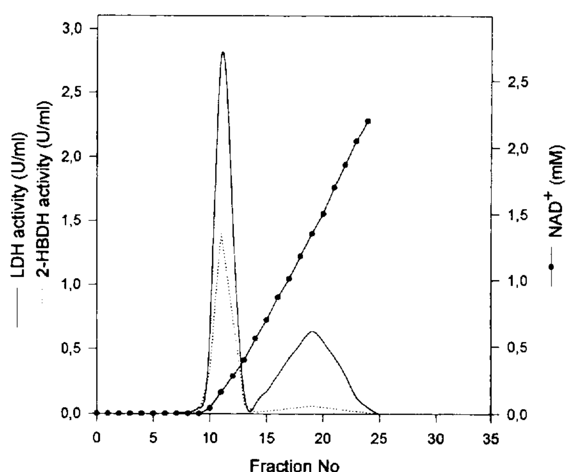

Fig. 9. Affinity chromatography of a mixture of lactate dehydrogenase isoenzymes from rabbit heart (LDH-B_4) and muscle (LDH-A_4) on Procion Blue H-5R–Sepharose 4B. The column (1.5×30 cm) was equilibrated with 50 m*M* phosphate buffer, pH 7.5 at 25°C. The dialyzed isoenzymes (5 units of LDH activity each) were applied. The enzymes were desorbed by a linear gradient of NAD^+ (0–3 m*M*) in equilibration buffer. The LDH as well as the 2-HBDH activity were determined in all fractions (1 ml). (From Ref. [105] with permission.)

of NADH (0–0.5 m*M*) in the following order, LDH-B_4, B_3A, B_2A_2, BA_3, A_4 as shown in Fig. 8 [65].

Another affinity matrix, oxamate–Sepharose, is also capable of separating isoenzymes of LDH. Due to different kinetic properties (the B-type isoenzyme but not the A-type is characterized by substrate inhibition) a so called "abortive complex" of enzyme–NAD^+–substrate is formed which causes stronger retardation of LDH-B_4 on columns with immobilized oxamate. A complete separation of LDH-A_4 from the other forms of LDH has been reported [63]. Exploiting screening results of affinity partitioning with diverse dye–ligands, the reactive dye Procion Blue H-5R has been selected to distinguish between LDH-A_4 and LDH-B_4 [105]. Both enzyme forms were bound to Procion Blue H-5R–Sepharose columns but can be separated from each other completely by applying a gradient of NAD^+ in the elution buffer (Fig. 9). This behaviour is due to differences in the relative affinity of both isoenzymes to the dye–ligand. Similar results have been reported by Lowe et al. using immobilized Cibacron Blue F3G-A in a HPLC mode [78].

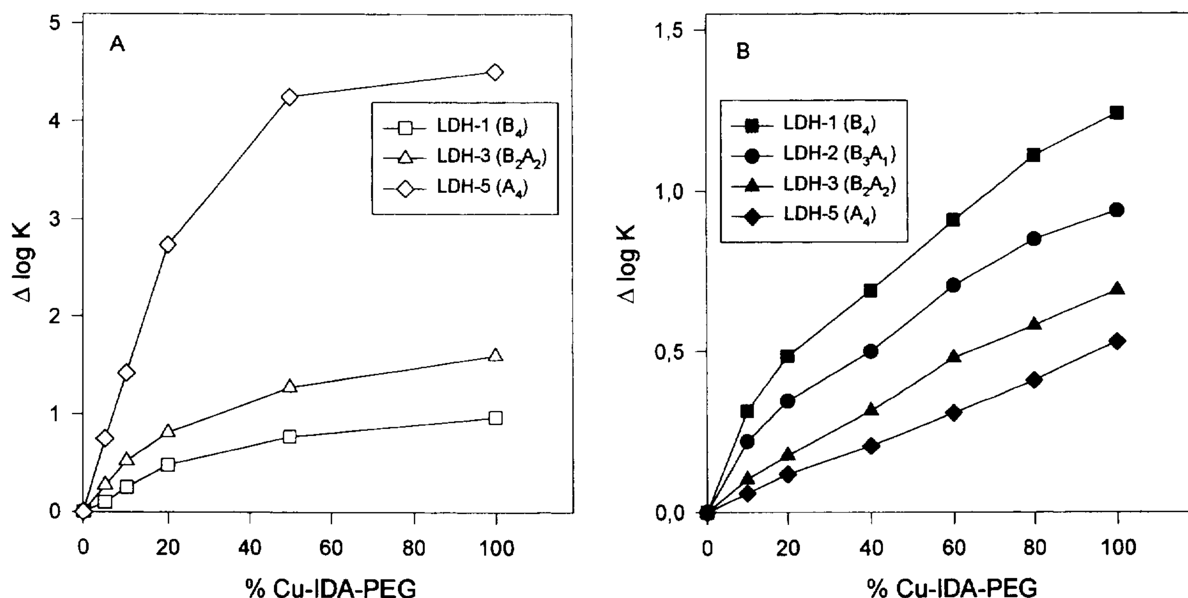

Fig. 10. Metal chelate affinity partition of human and rabbit LDH isoenzymes. (A) 5 to 10 units of purified rabbit isoenzymes were partitioned in two-phase systems (2g) composed of 10% PEG 1550, 10% Na_2SO_4, 2% PEG 6000 (or increasing substituting concentrations of Cu–IDA–PEG 6000) and 0.01 *M* sodium phosphate buffer, pH 7.0. The concentration of Cu–IDA–PEG is expressed as percentage of the total PEG 6000 which is replaced by Cu–IDA–PEG. Partition was performed at 22°C. (B) 2 to 6 units of purifed human isoenzymes were partitioned in two-phase systems as described in (A). (From Ref. [106] with permission.)

Metal chelate affinity partitioning has also been shown to distinguish different isoenzymes of LDH [106]. The principle of selection is based on the primary structure because the binding of proteins to transition metal chelates is histidine mediated (see Section 4.1.2.

Fig. 10 illustrates the effect of immobilized copper (Cu–IDA–PEG) on the partitioning of isoenzymes of rabbit and human LDH in a PEG/salt two-phase system. In the absence of the metal ion, the partition coefficient, K, did not vary strongly among the isoenzymes, which were concentrated favourably in the lower phase. The increase of the partition coefficient of rabbit LDH-A_4 of four orders of magnitude at high concentrations of the ligand–PEG (the K-value changed from 0.01 to about 500) in comparison to LDH-B_4 indicates differences of accessible histidine residues on the surface of rabbit LDH. The increments in the affinity of human LDH isoenzymes in a Cu–IDA system were found to be proportional to the number of B-type subunits in the tetrameric molecule. The overall lower binding of human LDH to copper compared with the rabbit isoenzymes implies a reduced number of metal chelate binding sites. The human A- and B-types possess only seven histidines [107]. Hence, a $\Delta \log K$ that is near to the value of rabbit LDH-B_4 can be expected. However, despite similar histidine content, fine differences in the metal chelate binding properties of the human isoenzymes were confirmed. The similarity of the amino acid sequence of the human A- and B-type is about 75%. Out of seven histidines, only one position is different, the B-type contains His_{156}, and the A-type His_{215}. The different affinity of human LDH to Cu–IDA–PEG could arise from the different availability of these histidine residues.

4.3. *Purification of lactate dehydrogenase from microbial sources and plants*

LDH (EC 1.1.1.27) was purified and characterized from many bacterial species, particularly in the genera *Streptococcus* [27,28,108–110], *Lactobacillus* [38,108,111], *Leuconostoc* [108] and *Pediococcus* [108] which are known as lactic acid bacteria.

A number of purification protocols for microbial LDH comprise steps such as ion-exchange chromatography and affinity chromatography applying AMP [38], oxamate [37] and dyes [27,29,38] as affinity ligands, respectively.

LDH from plants like potato tubers [112], roots [29] and lettuce [40] have been purified and well characterized.

5. Clinical significance

5.1. *Standard assay conditions*

The determination of serum LDH has been used for diagnostic purposes for the last 30 years. Although human LDH is widespread in most of the tissues and decreases in the following order: kidney > heart > skeletal muscle > pancreas > spleen > liver > lung > placenta [26], the enzyme activity found in serum or plasma usually originates from liver, heart, skeletal muscle, erythrocytes and tumours. LDH activity in other body fluids such as cerebrospinal fluid, effusions and urine has only limited clinical significance [113].

To obtain serum samples, the blood should be collected from a vein without stasis and large haemolysis must be prevented in any case. Plasma can also be used but the addition of oxalate or heparin for prevention of the blood clotting is not recommended. Due to a small lysis of erythrocytes during coagulation, the activity of LDH in serum is regularly higher in plasma [114]. The serum enzyme is stable for a period of 48 h at 4°C and at 20°C, respectively. The activity decreases after freezing depending on the isoenzyme pattern [115] (see also Section 3).

The determination of LDH activity in serum or plasma by continuously monitoring the consumption of NADH, while pyruvate is converted to lactate, is generally accepted with slight variations in the reaction conditions by most of the European Societies for Clinical Chemistry (see Table 7) [26,116]. However, neither a reference method of the International Federation of Clinical Chemistry, the European Committee for Clinical Laboratory Standards nor an international standard have been declared so far. Optimum substrate concentrations for the LDH assay in both reaction directions depending on the temperature are described [26,117,118].

Alternatively, colorimetric assays have been

Table 7
Recommended methods for the determination of total LDH activity in human serum [26,116]

Temperature	Buffer (m*M*)	pH	Pyruvate (m*M*)	NADH (m*M*)	EDTA (m*M*)	NaCl (m*M*)	Society
25°C	Phosphate (50)	7.5	0.6	0.18	–	–	DGKC
37°C	Tris (50)	7.4	1.2	0.15	5	–	SCE
30°C	Tris (80)	7.2	1.6	0.20	–	200	SFBC
30°C	Tris (50)	7.4	1.2	0.15	5	–	NVKC
30°C	Phosphate (65.4)	7.4	1.2	0.20	–	–	ACB

ACB: Assocation of Clinical Biochemists (British).
DGKB: Deutsche Gesellschaft für Klinische Chemie.
NVKC: Nederlands Vereniging voor Klinische Chemie.
SCE: Scandinavian Committee on Enzymes.
SFBC: Société Francaise de Biologie Clinique.

elaborated using phenazine methosulfate [119] and NADH/lipoamide oxidoreductase (EC 1.6.4.3) [120] as well as 3-[4,5-dimethylthiazolyl-(2)]-2,5-diphenyl-2H-tetrazolium bromide and a so called NADH/dye-dehydrogenase [42]. The determination of LDH activity by measuring the heat of the reaction applying microcalorimetric was also described [21]. According to Cai et al. [121], LDH activity can be measured by following the electrochemical oxidation of NADH at a modified microband gold electrode.

5.2. Isoenzyme detection in clinical practice

The importance of the analysis of the isoenzyme pattern is based upon their emergence during evolution and their functional significance in existing species. Isoenzymes are linked with cell differentiation and development, with metabolic regulation and tumour growth. The detection of tissue-specific forms of human LDH isoenzymes improves the diagnostic value of this enzyme significantly.

Differences in the distribution of LDH isoenzymes in tissues result in variations in the serum isoenzyme patterns according to the source of the enzyme. Since the serum contains varying proportions of the five isoenzymes, each with different optimum substrate concentrations, any single assay does not yield sufficient results. For example, the optimum concentration of pyruvate for LDH-5 (A_4) causes inhibition of LDH-1 (B_4) [26]. To minimize the differences in the kinetic properties of the LDH isoenzymes towards pyruvate, addition of NaCl to the assay at 30°C was recommended [26,122].

For the determination of LDH isoenzymes many approaches have been elaborated, either by employing separation techniques based on differences in the charge of the isoenzymes or by applying nonseparating methods founded on differences in kinetic, physico-chemical or immunological properties. For the separation of LDH isoenzymes in clinical practice, electrophoretic methods under slightly alkaline conditions [123] and anion-exchange chromatography [50,124] have found employment.

Because of the highly reliable results, the electrophoretic determination of the isoenzyme pattern is preferentially used. There are three main groups which suggest their clinical significance: (1) the anodic group in which LDH-1 (B_4) and LDH-2 (B_3A_1) predominate and which is found in erythrocytes, cardiac muscle and kidney; (2) the cathodic group with LDH-4 (B_1A_3) and LDH-5 (A_4) dominating originates from liver, skeletal muscle and neoplastic tissues; (3) the intermediate group, in which LDH-3 (B_2A_2) predominates, comes from lymphatic tissue, platelets and many malignant tissues [50]. In the serum of mammals, LDH-C_4 exhibits an electrophoretic mobility between LDH-3 and LDH-4 [51]. The electrophoretic separation of LDH isoenzymes in combination with fluorescence densitometry of the generated NAD^+ provides high sensitivity in assessing the complete isoenzyme pattern [50,123].

The ion-exchange chromatography was elaborated for LDH separation with various modifications in-

cluding batch and minicolumn procedures, salt gradient elution and in the HPLC-mode [125] (for review see [104]).

Non-separating detection methods for LDH isoenzymes are based on differences in the kinetics or in the heat and urea stability as well as in the immunological properties [104]. The fast moving fraction (LDH-1 and LDH-2) is more heat resistant than the slow moving one [126]. Urea inactivated liver LDH completely, whereas the heart activity was partially preserved [126]. Bernstein and Scinto [127] developed an assay for LDH-1 and LDH-2 with pyruvate at pH 7.1. Another method is founded on differences in the stability of LDH isoenzymes at alkaline pH [128]. Tanishima et al. [129] reported a procedure for the determination of LDH-1 and LDH-2 using 1,6-hexanediol as an inhibitor of the M-subunit. LDH isoenzymes containing the M-subunit are also inhibited by sodium perchlorate [130] and guanidine thiocyanate [131].

Rosalki and Wilkinson [132] found that the anodic LDH fraction is capable of reducing 2-oxobutyrate more rapidly than the cathodic group. This activity of LDH is called 2-hydroxybutyrate dehydrogenase or 2-HBDH-activity and is stable in serum for at least one week at 18°C. LDH-C_4 also possesses considerable 2-HBDH activity [50].

Most of the immunological techniques are based on the properties of antibodies against the M- or H-type. Both antibodies complex exclusively with its corresponding antigen and do not cross-react with eachother. Therefore, immunoinhibition techniques allow the determination of the residual LDH-1 and LDH-5 activity, respectively. Also routine radio- and enzyme-immunoassays for the determination of individual subunits have been developed [104,133].

5.3. Abnormal isoenzyme pattern

Abnormal electrophoretic mobilities of LDH isoenzymes based on the complex formation of the enzyme with immunoglobulins and the surface antigen of hepatitis B virus have been described [104]. Cabello et al. [134] reported on patients having an additional band on the cathodic side of LDH-5 which showed differences in the kinetic behaviour. This extra band was designated as LDH-6 and was characterized as a useful marker for severe liver injury indicating poor prognosis [104].

5.4. Genetic deficiencies and variants

Genetic deficiencies in either B- or A-subunits in the Japanese population has been well documented [135,136]. Both heterozygotes and homozygotes exist. The complete absence of the B-subunit appears to have no deleterious effect on health whereas the absence of the A-subunit generates a serious syndrome [9]. Diverse variants of the B- and A-subunits were described. All these variants can be grouped into slow and fast moving types [104]. In 1981, another kind of LDH isoenzymes was reported and designated as LDH_K [137]. This isoenzymic system is active in various malignant tissues and shows significant differences in molecular size and kinetic properties to the "classical LDH" (EC. 1.1.1.27) but structural similarity to the D-(−)-lactate specific enzyme (EC. 1.1.1.28) [104].

An unique electrophoretic form of LDH has been reported and designated as LDH-Z [104]. It occurs in early placenta and human choriocarcinoma and may be indicative of the trophoblastic origin of the cells.

5.5. Enzyme activity and isoenzyme pattern in diseases

5.5.1. General aspects

Acute diseases, generating a rapid release of the enzyme into the circulation, are associated with much higher LDH activity in serum than the chronic ones. The highest activities were found in patients with megaloblastic anaemia or malignancy.

The determination of the total serum LDH activity has only moderate diagnostic and differential-diagnostic value. Total levels of serum LDH activity should regarded in conjunction with other diagnostic parameters such as activity of alanine aminotransferase (EC. 2.6.1.2), aspartate aminotransferase (EC. 2.6.1.1), glutamate dehydrogenase (EC. 1.4.1.3), creatine kinase (EC. 2.7.3.2) and the concentration of myoglobin, troponin T and others. When LDH alone is increased in serum, liver and muscle tissues can be excluded as source. If a recent myocardial infarct can be neglected, the most likely causes for the increase of LDH activity in serum are haematological or malignant.

The determination of isoenzyme level and isoenzyme pattern improves the diagnostic value significantly. However, one has to take into considera-

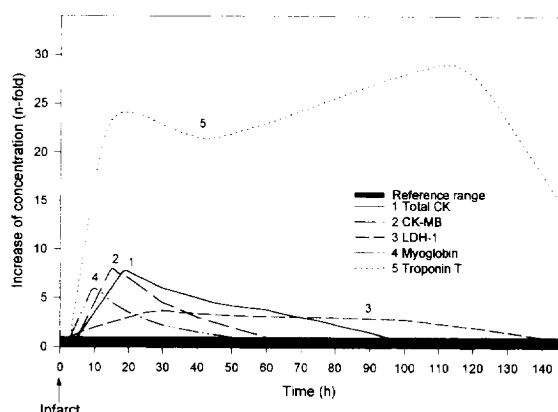

Fig. 11. Dependence of the concentration in serum of diagnostic parameters of myocardial infarct on time after onset of symptoms (From Ref. [139] with permission).

tion the fact that LDH isoenzymes are cleared from the circulation at different rates. For example, the half life time of LDH-1 is about 113 h and that of LDH-5 is only 10 h [116].

5.5.2. Indications

Myocardial infarct. For the diagnosis of myocardial infarct, the determination of LDH-1 is most widely used. The activity of the isoenzyme starts to rise in serum within 6–20 h after onset of clinical symptoms. Maximum activity appears at 36–60 h and remains elevated for 6–11 days. However, other parameters like myoglobin, creatine kinase isoenzyme (CK–MB) and troponin T [138,139] seems to be of higher value as shown in Fig. 11. The determination of LDH-1 is valid for the confirmation of suspicions of a myocardial infarct when the appearance of clinical symptoms was more than 2 days later and for the evaluation of the process. But, nonischaemic heart diseases (inflammatory processes) generate a similar increase of LDH activity and isoenzyme pattern [140].

Liver diseases. As a rule, the activities of aspartate aminotransferase (EC. 2.6.11), alanine aminotransferase (EC. 2.6.1.2) and glutamate dehydrogenase (EC. 1.4.1.3) in serum are used as diagnostic parameters for liver cell damage [141]. The increase of LDH-5 was found as an indicator of hepatocellular damage (viral hepatitis, drug toxicity, tumours) and is also valid for the differentiation of the icterus [104,116]. However, the short half-life time of LDH-5 in serum (10±2 h) and the insufficient estimation of the degree of the cell damage, because LDH is localized only in the cytoplasm, restrict the diagnostic value of LDH for liver diseases.

Haematological diseases. Since erythrocytes contain large amounts of LDH, every event generating a haemolytic process (haemoglobinopathies, megaloblastic and pernicious anaemia) increases LDH serum activity. Mature erythrocytes exhibit an isoenzyme pattern with LDH-1, LDH-2 and LDH-3 whereas immature nucleated erythrocytes possess more LDH-5 [104,116].

Skeletal muscle diseases. The LDH isoenzyme pattern switchs depending on the disease. LDH-5 is increased in relation to inflammatory disorders, the release of LDH-1/LDH-2 is amplified after outbreak of muscle dystrophy [104,116].

Malignancy. There are differences in the isoenzyme pattern in tumours and in the surrounding normal tissue. In most tumours, the pattern is shifted towards the cathodic LDH-4 and LDH-5. High serum levels of LDH at initial presentation or pretreatment predict metastasis or short survival in Hodgkin's disease, histiocytic lymphoma, Burkitt's lymphoma and Ewing's sarcoma. When other reasons can be excluded, the increase of LDH-1 is a useful marker for germ cell carcinoma [142,143]. However, LDH is not recommended as marker enzyme for malignant diseases [144].

Miscellaneous conditions. Other diseases which may lead to an increase of serum LDH activity are asthma, infectious mononucleosis, measles, renal disorders, myeloproliferative syndrome and hypothermia etc. [104,116]. The difficulties and the significance of the determination of LDH activity in cerebrospinal fluid, effusions and urine have been discussed in the literature [116,145,146].

The amount of the isoenzyme LDH-C_4 was related to the testicular maturity. Some forms of testicular dysfunction were associated with its disappearance. Thus, the quantitative determination of LDH-C_4 activity in seminal plasma has been developed [10,55,104,147].

6. The significance of lactate dehydrogenase as an auxiliary enzyme in enzymatic assays

Commercial LDH from muscle or heart is preferably used for the quantitative determination of various metabolites like L-alanine, citrate, creatine, creatinine, succinate, triglycerides, lecithin, D-sorbitol, sialic acid, glycerol, D-glycerate, L-(+)-lactate and pyruvate [148–150]. The lactate/pyruvate-quotient is an important parameter in the diagnosis of peripheral hypoxia (shock, respiratory insufficiency). Moreover, the determination of lactate is important in clinical biochemistry indicating lactic acidosis or liver and kidney insufficiency and for the medical control of athletes in competitive sports. In addition to the clinical approach, the determination of lactate is also required in microbiology as well as in the paper, cosmetic and food industry [151]. The main disadvantage in the determination of L-(+)-lactate with LDH is the unfavourable equilibrium preferring the formation of lactate (see Section 2). Several approaches have been tried to circumvent these difficulties like the use of alkaline media, different reagents to trap the reaction product pyruvate [135] or the use of an NAD^+-independent yeast LDH (EC. 1.1.2.3). This flavohaemoprotein transfers hydrogen from L-(+)-lactate to ferricytochrome c. As electron acceptors hexacyanoferrate(III) or Fe(III)-phenanthroline were used [152]. Also an enzyme electrode for lactate determination with continuous reoxidation of NADH at a platinum electrode was constructed [153].

LDH is a common auxiliary enzyme for the determination of many enzymes like alanine aminotransferase, ATPases, adenylate kinase and pyruvate kinase [148].

7. Lactate dehydrogenase and cell injury

There is no evidence that cells actively secrete LDH or that they benefit from its loss. Therefore, it is accepted that LDH release from cells is a pathological manifestation of a defect in the plasma membrane which acts as a permeability barrier. The release of LDH activity into the surrounding medium was also used to monitor tissue and cell damage in vitro. In a wide variety of cell cultures including myocytes, hepatocytes, platelets, fibroblasts, macrophages, erythrocytes, lymphocytes, spermatozoa, HeLa cells, Chang liver cells and others, the loss of LDH has been found as an indicator of cell damage. Although the LDH release into the perfusate or culture fluid has been frequently used as a measure of cell injury of heart, liver, kidney, cartilage, etc., it is not always clear what this release really indicates, the extent of damage or the survival of the cell [154].

8. List of abbreviations

ACB	Association of Clinical Biochemists (British)
CH-Sepharose	Carboxyhexyl-Sepharose
DEAE-	Diethylaminoethyl-
DGKB	Deutsche Gesellschaft für Klinische Chemie
2-HBDH	2-Oxobutyrate dehydrogenase
HEPES	H-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IDA	Iminodiacetate
IMAC	Metal ion affinity chromatography
LDH	Lactate dehydrogenase
NVKC	Nederlands Vereniging voor Klinische Chemie
QAE-	Quaternary aminoethyl-
SCE	Scandinavian Committee on Enzymes
SFBC	Société Francaise de Biologie Clinique
PEG	Poly(ethylene glycol)

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