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REVIEW

LACTATE DEHYDROGENASE ISOENZYMES

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LIST OF ABBREVIATIONS

AMI	Acute myocardial infarction
Bis-Tris	2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol
CK	Creatine kinase
CK-MB	Creatine kinase MB
CSF	Cerebrospinal fluid
DEAE	Diethylaminoethyl
HBD	α-Hydroxybutyrate dehydrogenase
HPLC	High-performance liquid chromatography
K_m	Michaelis constant
LD	Lactate dehydrogenase
MTT	Thiazolyl blue
NAD	Nicotinamide-adenine dinucleotide
NBT	Nitroblue tetrazolium
PCR	Post-column reactor
PMS	Phenazine methosulphate
QAE	Quaternary aminoethyl
Tris	Tris(hydroxymethyl)aminomethane

1. INTRODUCTION

Lactate dehydrogenase (LD) (L-lactate:NAD oxidoreductase, EC 1.1.1.27) and other enzymes have been resolved into several distinguishable molecular types which are called "isozymes" or "isoenzymes" [1]. LD is a tetrameric molecule, which is composed of two subunits designated H (heart) or B and M (muscle) or A [2,3]. The five isoenzymes are numbered (from LD-1 to LD-5) H_4, H_3M, H_2M_2, HM_3, M_4; H_4 has the highest and M_4 the lowest migration rate toward the anode. The relative molecular mass of each subunit is 34 000 and of each isoenzyme 134 000 [4].

The homotetramers, M_4 and H_4, differ in their Michaelis constants, K_m. LD-M_4 isoenzymes have relatively high K_m values for pyruvate [5,6]. Moreover, LD-H_4 isoenzymes are more sensitive to inhibition by high pyruvate concentrations than are M_4 isoenzymes, which are relatively indifferent to substrate concentration, at least in physiological ranges [2,7]. These characteristics have generally been used to explain the physiological role and tissue distribution of the isoenzymes.

The determination of LD isoenzymes was the first routine isoenzyme technique to be widely adopted by clinical laboratories. The first biological application of LD heterogeneity utilized the technique of starch block electrophoresis [8], but methods other than electrophoresis, including chromatography and the immunochemical method, have subsequently been successfully employed. Electrophoretic technique affords the capacity for separating multiple specimens si-

multaneously. These features facilitate comparative studies of the LD isoenzymes present in different biological materials.

LD is an enzyme present in all tissues. Consequently, elevations of the total enzymatic activity in serum reflect no specific damage of a single tissue or organ. Additional information can be obtained through the separation and quantitation of LD isoenzymes. Alterations in the serum LD isoenzyme pattern have been employed to indicate the site of pathological involvement. This paper reviews LD isoenzymes mainly with respect to the clinical laboratory and laboratory medicine.

2. METHODS FOR ISOENZYME ANALYSIS

Many techniques have been used for separating the LD isoenzymes. Electrophoretic methods on various media have been the most popular, column chromatography has been used because of its relative speed and a rapid immunochemical technique for the assay of LD-1 alone has recently been published. The techniques used for the separation and quantitation of LD isoenzymes can be divided into two categories. The first category offers the possibility of separating all five isoenzymes, e.g., by electrophoresis [9–22] or chromatography [23–41], or of detecting some abnormal LD isoenzymes. The other approach is to quantify LD-1, or LD-1 and LD-2 only. This approach is applied mainly for the diagnosis of acute myocardial infarction (AMI) and is based on differences in the physical or kinetic properties of the H and M subunits, such as substrate specificity [42–46], heat denaturation [47], stability in urea [48] or at alkaline pH [49,50], inhibition by pyruvate [51], oxamate [52], oxalate [52] or 1,6-hexanediol [53,54], differences in Michaelis constant [5,7] and differences in the immunological properties of the LD isoenzymes [55–63].

2.1. Electrophoresis

2.1.1. Sample preparation

LD isoenzymes must be analysed in a fresh specimen. The use of serum rather than plasma avoids the possibility of isoenzyme inhibition by anticoagulants [64]. Other body fluids may require pre-treatment with thrombin before separation of the LD isoenzymes [12]. Human tissue is cut into small pieces and homogenized in 0.02 *M* tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (pH 7.4). The homogenate is centrifuged (100 000 *g*, 60 min, 4 °C) and the supernatant fluid is analysed.

The electrophoretic method can be satisfactorily applied to samples having an LD activity as low as half of the lower limit of normal serum LD activity, and the activity of the sample should not exceed the upper limit of the normal serum LD activity. Therefore, it is necessary to determine the total enzyme activity of the sample prior to sample application. Moreover, it is necessary to assume that the total enzyme activity may be concentrated in a single isoenzyme fraction, and the volume or dilution of the sample to be applied should be adjusted accordingly. Whereas in normal sera, and some pathological sera, LD activity may be spread over several fractions, in other pathological sera the activity may be concentrated

in only one or two fractions, and this must be allowed for if the relationship between the staining capacity and isoenzyme activity is not to be invalidated. Substrate depletion over the most active LD isoenzyme bands will result in erroneous determination of the isoenzymes [10,12,65]. Finally, haemolysed specimens should not be used for LD isoenzyme determinations because of the very high LD-1 and LD-2 content of red blood cells.

2.1.2. Separation

The charge on each subunit differs, thus imparting a different total charge to each isoenzyme. Supporting media for electrophoretic separations include starch [6,9,10], paper [11], agar–agarose [10,12–15], polyacrylamide gel [17,18] and cellulose acetate [19–22]. Of these media, agarose gel and cellulose acetate membranes are widely employed in the clinical laboratory. Separation conditions, including voltage and current levels, liable to produce heating, should be avoided during the separation in order to prevent the possibility of enzyme inactivation, particularly of heat-sensitive slow-moving isoenzymes (LD-4 and LD-5). Separations should therefore be carried out with adequate cooling to 4°C [64]. The buffer used for electrophoretic separation must give an adequate isoenzyme separation without enzyme inhibition. The barbiturate buffer (pH 8.6) is the most popular for protein and isoenzyme analysis, including LD.

2.1.3. Detection

The relative amounts of each isoenzyme present have been quantitated by spectrophotometric [9,11], colorimetric [10,17–22] and fluorometric [12–16] methods. The colorimetric method uses a staining solution containing lactate, nicotinamide adenine dinucleotide (NAD), tetrazolium salts [nitro blue tetrazolium (NBT), thiazolyl blue (MTT), etc.] and phenazine methosulphate (PMS) or diaphorase. LD catalyses the following reaction:

L-lactate + NAD → pyruvate + NADH (fluorescence of NADH)

The NADH generated is detected by fluorometry or used to reduce the tetrazolium salt to a coloured insoluble formazan:

NADH + MTT + PMS → NAD + insoluble formazan (colour of formazan)

Densitometric scanning of the resultant pattern yields the relative percentages of the isoenzymes present.

The concentrations of individual constituents of the LD isoenzyme mixture show considerable variations from the published literature. One reason may be that the concentration of L-lactate which yields optimal activity for fast-moving LD isoenzymes (LD-1 and LD-2) is lower than that required to achieve optimal activity with the slow-moving LD isoenzymes. This phenomenon produces a significant effect on the LD isoenzyme pattern [64,66]. Higher concentrations of L-lactate lead to relatively higher activity of the slow-moving isoenzymes [66]. Fig. 1 shows relative proportions of LD-2, LD-3, LD-4 and LD-5 against LD-1 and the corresponding LD isoenzyme pattern dependence on lactate concentration.

In our laboratory, the staining solution contained 0.2 *M* lithium lactate (1 ml),

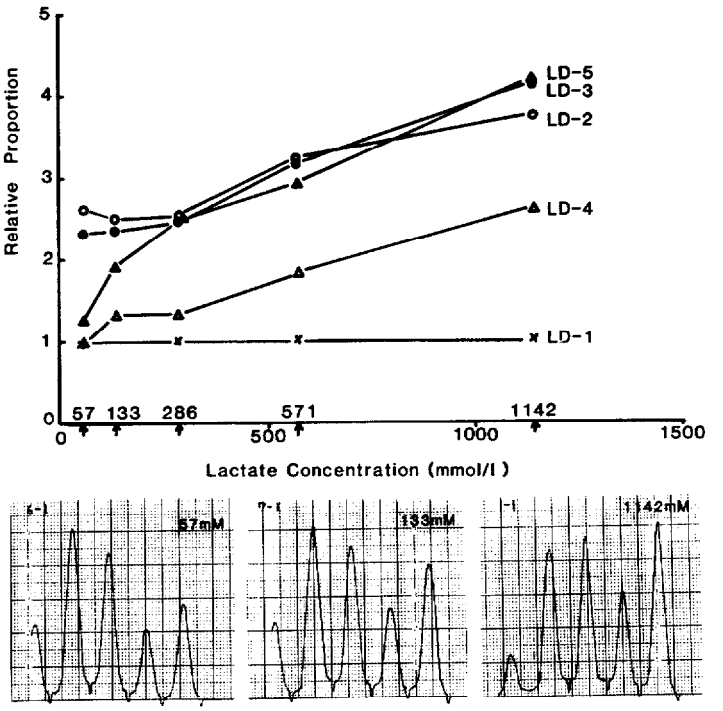

Fig. 1. Effects of lactate concentration in the staining solution on LD isoenzyme pattterns. Upper panel: effect of lactate concentration on the relative proportions of individual isoenzymes (ratio of each isoenzyme to LD-1). Lower panel: Typical densitometric profiles at three different lactate concentrations. Higher concentrations of lactate lead to relatively higher activity of slow-moving isoenzymes. The staining conditions were as described in ref. 21.

10 mg/dl NAD (0.1 ml), 10 mg per 10 ml MTT (0.25 ml) and 10 mg/dl PMS (0.15 ml). All reagents were dissolved in 0.06 *M* Tris–HCl buffer (pH 7.4) [21]. McKenzie et al. [12] proposed a selective method of detecting LD isoenzymes. Their detection solution contained 0.5 *M* lithium lactate and 7.5 m*M* NAD in doubly distilled water (pH adjusted to 7.0 with 100 m*M* hydrochloric acid).

The optimum staining temperature is 37°C. At lower temperatures the colour intensity is too low, whereas at higher temperatures LD-5 is markedly inactivated and the background colour increases rapidly [64,67].

To obtain separations with a colourless background, the staining technique and incubation time are important. Here one of the critical factors is to optimize the equilibrium of the lactate–pyruvate system, which depends on the rate of hydrogen removal effected by PMS. This hydrogen acceptor is very unstable, particularly at higher pH [68]. In addition, control of the staining reagents, especially PMS, is important.

McKenzie and Henderson [69] and Pridgar et al. [16] have shown that peak-area (integration) measurements offer more accurate, although less precise, data than peak-height (amplitude) measurements.

Typical patterns for LD isoenzymes analysed with electrophoretic separation

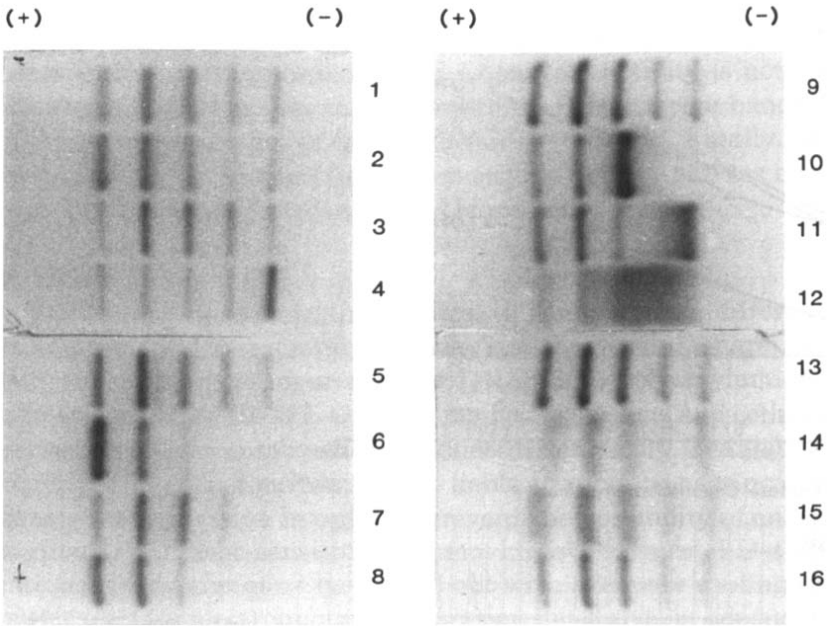

Fig. 2. Examples of LD electrophoretic patterns on cellulose acetate membranes. From anode (left) to cathode (right). From LD-1 to LD-5: 9, 13, 16, controls for isoenzyme mobility; 1, acute lymphoblastic leukaemia; 2, liver cirrhosis; 3, colonic cancer; 4, acute hepatitis; 5, extra band produced by skin tumour (positioned between LD-2 and LD-3); 6, acute myocardial infarction; 7, muscular dystrophy; 8, haemolytic anaemia; 10, LD-immunoglobulin A (kappa) complex; 11, LD-immunoglobulin G (lambda) complex; 12, LD-immunoglobulin G (kappa, lambda) complex; 14, H subunit variant; 15, M subunit variant.

using Cellogel membrane and colorimetric staining in our laboratory are shown in Fig. 2.

2.2. Chromatography

Several column chromatographic methods have been described [17,23,24] for the separation of LD isoenzymes, most of them tedious and not suitable for routine use. Mini-column systems were originally reported for use in partial separations [25–30] of LD isoenzymes. The main purpose of these methods was in the interpretation of LD isoenzyme ratios for diagnostic purposes in AMI. More recently, miniature ion-exchange column methods have been employed for the separation of all five LD isoenzymes in human sera [29,30]. The separations are carried out with anion-exchange columns and individual isoenzymes are eluted at different salt concentrations. LD-5 is eluted first, with LD-1 being eluted at the highest salt concentrations. Either stepped or continuous gradients elute the individual isoenzyme fractions. The separated fractions are assayed by the usual method for LD activity. A survey of LD isoenzyme analysis by chromatography is summarized in Table 1.

Mercer [25] reported that the electrophoretic values for LD isoenzymes, especially LD-3, LD-4 and LD-5, were 20% lower than the chromatographic values.

According to Menon et al. [30], the values for LD-3 are higher whereas the LD-4 and LD-5 values are lower in electrophoretic assay compared with chromatographic data. On the other hand, Hsu et al. [29] reported results indicating that the column method yielded higher values for LD-1 and LD-2 and lower values for LD-5 compared with electrophoresis. This probably results from the differences in the LD assay methods used in the two procedures [29]. Therefore, for any diagnostic interpretation within a specific population group it is necessary to relate the results to the method used.

Medium-to-low pressure column chromatography has not been used in routine clinical analytical work for separating LD isoenzymes so far. Modern high-performance ion-exchange liquid chromatography, however, has been developed for LD isoenzyme analysis in serum [33–41] and has achieved an important position in the clinical laboratory. Excellent reviews of isoenzyme analysis by high-performance liquid chromatographic (HPLC) techniques have been published [33,34]. The separation of LD isoenzymes is based on differences in electrostatic interactions which largely govern the ion-exchange process. A gradient pumping system is generally required to separate isoenzymes by ion exchange. For example, the buffers for gradient elution were 0.02 *M* Tris–HCl (pH 7.8) (buffer A) and 0.02 *M* Tris–HCl containing 0.15 *M* sodium chloride (pH 7.8). The gradient was begun automatically 2 min after injection. It was advanced at a rate of 3%/min for 4 min, 6%/min for 2 min and 3%/min until the end of the gradient. Kawaguchi et al. [40,41] obtained a good resolution and short analysis time using a five-step linear gradient. The elution conditions were as follows: solvent A, 0.01 *M* Tris–HCl buffer (pH 8.0); solvent B, 0.5 *M* sodium chloride in solvent A; starting eluent, 7% solvent B; linear gradients 7–9% solvent B in 0.7 min, 9–20% solvent in B in 0.5 min, 20–26% solvent B in 1.8 min, 26–33% solvent B in 3.3 min and 33–70% solvent B.

The isoenzyme profiles obtained were analysed by computer programs which were available for both absorbance and fluorescence detection. For the detection reagents, 400 m*M* Tris–HCl buffer (pH 8.7) containing 70 m*M* L(+)-lactate, 4 m*M* NAD and 0.1% Brij-35 were used. The effluent was mixed with this reagent and incubated for 3 min at 37°C in a reaction coil; NADH formed in this reaction was quantified fluorimetrically at 370/465 nm [40,41].

Chang et al. [35] first introduced a post-column reactor (PCR) based on HPLC pumps and developed the continuous detection of LD isoenzymes (Fig. 3A). Later, Schroeder et al. [36] reported another version of continuous detection for monitoring the activity of isoenzyme eluates. Using a single PCR detection system, optical interferences in serum samples and baseline drift, especially at the end of the gradient, caused many problems in data interpretation. To overcome these problems, additional plumbing and an additional detector are required. A dual-detector PCR operating in the parallel mode was introduced (Fig. 3B). In the system, identical flow-rates could not be maintained as a result of the temperature-dependent viscosity difference between channels. However, this problem was surmounted by using a stream-switching valve to divert the stream alternately between channels [38] (Fig. 3C). Fulton et al. [37] used a serial-stream, dual-

TABLE 1

CHARACTERISTICS OF CHROMATOGRAPHIC SEPARATIONS USING MINICOLUMN SYSTEMS

Reference	Ion-exchange resin*	Elution principle*	Separated isoenzyme
Mercer [25]	DEAE-Sephadex A-50 (Pharmacia)	Stepwise elution with 0.05 *M* Tris–HCl buffer (pH 8.0) containing NaCl (0.1 and 0.2 *M*)	LD-1, LD-2, CK-MB
Lederer et al. [26]	DEAE-Sephadex A-50	Three-step batch elution: NaCl (0.5 *M*)	LD-1, LD-2, CK-MB
Mercer [27]	DEAE-Sephadex A-50	Stepwise elution with 0.05 *M* Tris–HCl buffer (pH 8.0) containing NaCl (0.1, 0.15, 0.2 *M*)	LD-1, LD-2
Hsu et al. [29]	QAE-Sephadex A-50 (Pharmacia)	Stepwise elution with five different buffers, 0.02 *M* Tris–HCl buffer (pH 7.4) containing five NaCl concentrations (0, 0.06, 0.1, 0.145, 0.24 *M*)	Five LD isoenzymes
Menon et al. [30]	DEAE-cellulose (Pharmacia)	pH–coupled salt gradient elution technique, five different buffers containing 0.02 *M* Tris–HCl with increasing salt concentration (0–0.25 *M*, NaCl) and decreasing pH (8.0–7.2)	Five LD isoenzymes
Morin and Barton [32]	AG-MP-1 (Bio-Rad Labs.)	0.1 *M* Bis-Tris acetate (pH 6.3) with 0.1 *M* magnesium acetate with a low-speed centrifugation	LD-1, CK-MB
Nathan et al. [28]	DEAE-Sephadex	Elution with water	LD-5

*Tris = tris(hydroxymethyl)aminomethane; Bis-Tris = 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; QAE = quaternary aminoethyl.

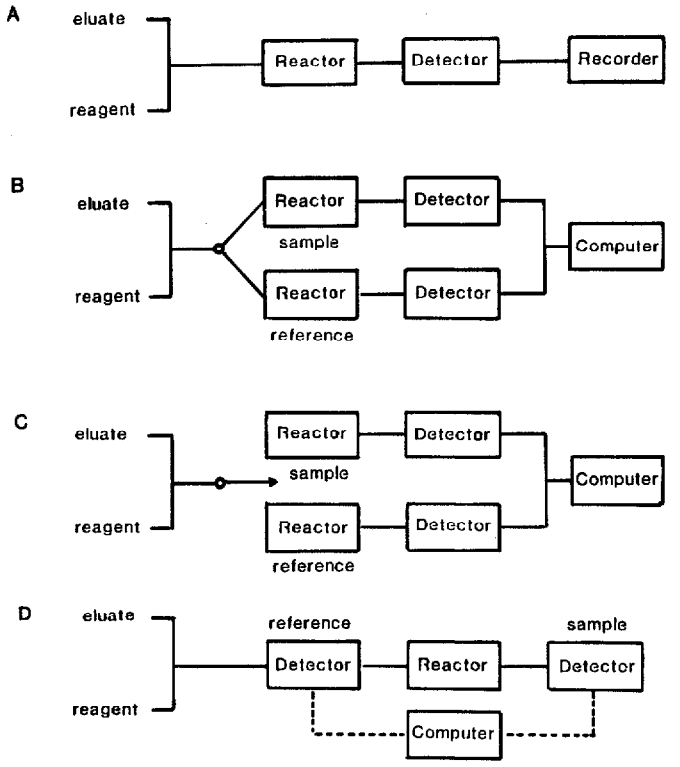

Fig. 3. Scheme of the detector in the HPLC system for the determination of LD isoenzymes. (A) Single-detector post-column reactor; (B) dual-detector post-column reactor, parallel mode; (C) parallel-stream dual-detector post-column reactor with stream switching. The switching valve diverts the stream for equal periods between the sample and reference channels and also synchronizes the readings of the two detectors. (D) Dual-detector, serial-mode post-column reactor. A computer performs a subtraction of the detector readings.

detector PCR with computerized data acquisition and reduction in order to improve the linearity and sensitivity (Fig. 3D).

Kawaguchi et al. [40,41] performed the repetitive analysis of serum LD isoenzymes by using a weak anion exchanger (TSK gel DEAE-5PW), which was developed by introducing diethylaminoethyl (DEAE) groups into TSK gel G5000PW (a hydrophilic polymer-based material of large pore size, manufactured by Toyo Soda), for HPLC. They also introduced this procedure into routine laboratory work. Fig. 4 shows the set-up of the HPLC system and Fig. 5 shows two chromatograms from the 1st and the 1000th run of the same column separation. Obviously, the resolution of the LD isoenzymes was maintained over a series of more than 1000 runs. LD isoenzymes were eluted by a five-step linear gradient of sodium chloride as described above. After elution with starting eluent for 0.5 min, the next sample can then be injected. By using this system, the analysis time was reduced to 10 min per sample.

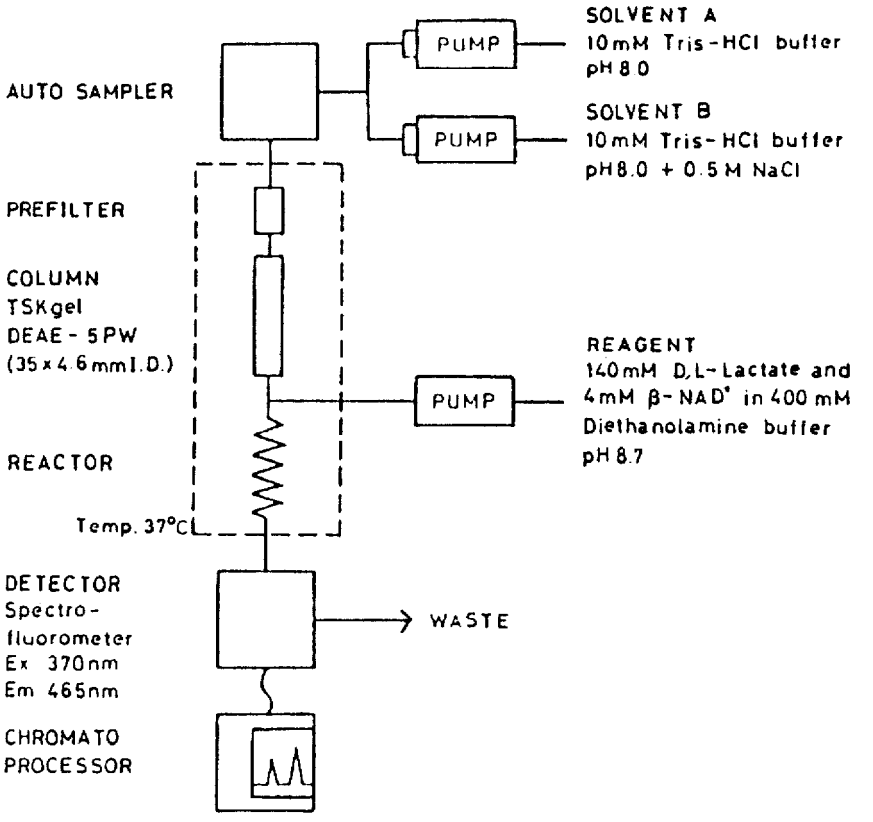

Fig. 4. HPLC system for the determination of LD isoenzymes. The column (35 mm×4.6 mm I.D.) contained TSK gel DEAE-5PW (10 μm particle diameter). Chromatographic conditions: solvent A, 0.01 *M* Tris–HCl buffer (pH 8.0); solvent B, 0.5 *M* sodium chloride in solvent A. The flow-rate was 1.6 ml/min and the sample volume (human serum diluted four-fold with solvent A) was 100 μl. Starting eluent was 7% solvent B. Linear gradients were 7–9% solvent B in 0.7 min, 9–20% solvent B in 0.5 min, 20–26% solvent B in 1.8 min, 26–33% solvent B in 3.3 min and 33–70% solvent B. (Reproduced with permission from ref. 41).

2.3. Immunochemical methods

Antibodies produced against LD-1 do not cross-react with LD-5, and vice versa. Nevertheless, as would be expected from their hybrid tetrameric structure, LD-2, LD-3 and LD-4 react with antibodies to give either LD-1 or LD-5 [4,55,56,70] to different extents. Usategui-Gomez et al. [57] described an immunochemical method allowing the specific quantitative determination of LD-1 activity. Generally, the immunochemical methods utilize antibodies against the M subunit of LD [57,58,62]. The complex between LD containing M subunit and anti-M antibody is allowed to react with a second antibody against anti-M antibody. Subsequent centrifugation removes all the LD isoenzymes containing M subunit and only LD-1 can be recovered in the supernatant. The remaining LD activity is measured by the usual method for LD activity. Pråhl et al. [61] used a biolumi-

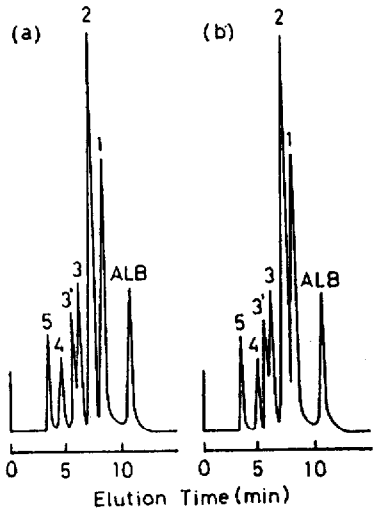

Fig. 5. Chromatograms from (a) the 1st and (b) the 1000th run of TSK gel DEAE-5PW separation. Experimental conditions as in Fig. 4. ALB-albumin. (Reproduced with permission from ref. 41).

nescent assay of LD-1 activity after the immunochemical isolation of LD-1. The assay procedure is sensitive and may be used for immunochemical separation.

The reagent kit for the immunochemical separation of LD-1 from the other four isoenzymes is available commercially (Roche Diagnostics). It consists of an anti-LD-5 goat-serum antibody and a second antibody suspension containing a polymer-bound anti-goat immunoglobulin from donkey serum. This method is generally considered to be a more sensitive indicator of early elevations in LD-1 after AMI [57–60,63,71–74]. Also, the development of an immunochemical method for LD isoenzymes for the centrifugal analyser has been reported [59,63].

Vaidya et al. [75] developed a quantitative assay for LD-5 in which LD-5 activity is measured after extraction of the other isoenzymes by making use of two monoclonal antibodies immobilized on latex beads. The procedure may be of value in the rapid clinical diagnosis of liver and skeletal muscle disorders, and in the detection of certain human carcinomas in which serum LD-5 activity is elevated.

Radioimmunoassay and enzyme immunoassay techniques have already been routinely applied to the determination of concentrations of H or M subunit of LD in AMI [76,77], severe traumatic coma [78], protein turnover rates [79] and genetic variants [80]. Catalytic assays will continue to occupy the predominant position in some situations and a combination of the two (enzyme activity and concentration) will provide new information in others.

2.4. Kinetic methods

Kinetic methods are based on the differences of LD isoenzymes in catalytic properties (application of substrate analogues) and differences in structural stability. Rosalki and Wilkinson [42] first demonstrated that LD-1 reduced 2-oxobutyrate more readily than did LD-5. When this substrate is substituted for

pyruvate (as the enzyme substrate), the reduction, which has been termed α-hydroxybutyrate dehydrogenase (HBD) activity [43], can be used as a convenient indicator of the presence of the fast-moving LD isoenzymes [42,43,46]. Therefore, the determination of HBD activity in serum has established itself in the diagnosis of AMI [42,46] and has been applied to continuous flow automated techniques [44,45].

The following differences in the structural stability of LD isoenzymes can also be expected for their assay. The fast-moving (cardiac) LD is more heat resistant than the slow-moving LD [6,47]. Plummer et al. [48] showed that urea completely inhibited the LD activity of human liver extract, whereas that of human heart was partially preserved. Recently, Bernstein and Scinto [51] developed a method for the assay of the activity of LD-1 and LD-2 by inhibition of the H subunit by pyruvate at pH 7.1. Takizawa et al. [49] reported a spectrophotometric method based on the different stabilities of LD isoenzymes to alkaline pH. The method was developed into a fully automated assay for LD-1 with the use of a centrifugal analyser after alkaline inactivation [50]. Tanishima et al. [53] reported a method for measuring LD-1 and LD-2 activity using 1,6-hexanediol as an inhibitor of the M subunit. They also used this inhibitor for automated determination [54].

3. ADVANTAGES AND DEFECTS OF THE METHODS

Every technique possesses different advantages and drawbacks. Of the numerous published and commercially available methods for separating and measuring LD isoenzymes, electrophoretic methods are generally preferred because the resulting pattern is directly observable and all five isoenzymes are resolved in a single procedure. Table 2 shows the properties of the four main procedures used for LD isoenzyme analysis.

Low-to-medium pressure ion-exchange chromatographic methods are quanti-

TABLE 2

PROPERTIES OF FOUR MAIN PROCEDURES FOR THE ANALYSIS OF LD ISOENZYMES

Method	Precision (%)	(C.V.)	Sample volume (μl)	Time (min)
Electrophoresis	Within-run	1.9–11.4	<5	60–90
	Between-run	3.4–21.8		
Minicolumn ion-exchange chromatography	Within-run	4.3	250–500	30–60
	Between-run	6.3		
HPLC	Within-run	0.8–14.3	10	10
Immunochemical method	Within-run	1.0– 5.8	60–200	30
	Between-run	3.1– 7.7		

tative and, if properly monitored, yield reasonable resolution. Their main disadvantages are dilution of the collected sample during elution (except in one method [32]) and the possibility that the isoenzymes are mutually contaminated owing to incomplete resolution. These defects in ion-exchange column chromatography can be overcome by introducing high-performance methods, which show increased speed, resolution, precision and accuracy [38,40]. Moreover, they can be automated; nevertheless additional technological developments are still needed in order to meet all demands put on a routine assay.

Immunochemical methods are attractive because they combine good sensitivity and quantification with simplicity. Ali et al. [60] reported that the technician's time required to perform the immunochemical method and electrophoretic analysis for LD-1 of 100 sera was 320 and 548 min, respectively. The time study of electrophoretic analysis included the time required for preparing LD electrophoresis densitometric tracings for mounting on the laboratory reports. Thus, the immunochemical method clearly saves the technician's time. The immunochemical LD-1 assay appears to be more sensitive than the creatine kinase (EC 2.7.3.2) (CK)-MB assay and appears to be an earlier indicator of AMI than CK-MB [58]. The problems of the interference from LD-2 and LD-3 that one encounters in kinetic methods involving heat stability, hydroxybutyrate as a substrate analogue or chemical inhibitors such as urea or excess lactate can be avoided. The main feature of the immunochemical methods is the high specificity of the antigen–antibody reaction. In addition, the same reagents and instruments that are routinely used in the laboratory to determine total LD activity can be used to determine LD-1.

The principal advantage of kinetic methods for determining LD isoenzyme content is their rapidity, their cheapness and their adaptability to automated instruments. The methods are, however, inferior to immunochemical methods in terms of their specificity because of interference from other isoenzymes.

Electrophoretic methods are generally simple to perform but are more time-consuming when analysing large series of samples (see above). In addition, unusual specimens may present interpretation problems. Considerable skill is required to obtain good electrophoretograms and meaningful analyses. Also, there are differences in the percentage of isoenzymes assayed by using different densitometers with identical electrophoresed samples [30]. These differences are, however, apparently smaller than differences which are due to staining solutions, electrophoretic support media and possibly other reasons. Electrophoretic isoenzyme analysis is most often used in the clinical context for the diagnosis of AMI. Although quantitation of LD-1 after electrophoresis offers an excellent additional method for the diagnosis of AMI, it has not been widely applied, probably because the test is time-consuming and requires relatively expensive instruments and specially trained personnel. The immunochemical assay for LD-1 activity after separation of the M subunit containing isoenzymes is less time-consuming and does not require trained personnel. Therefore, it is particularly suited as a preliminary test even for smaller laboratories.

Electrophoresis on occasion can provide additional information about elevations of total LD, especially in cases of muscle trauma or liver damage (with

increases in LD-5) and a variety of malignancies. In this respect, electrophoresis combined with densitometry is the most frequently used method for determining the distribution of LD isoenzymes in clinical laboratories. HPLC appears to be a useful method, although it requires further perfection and, consequently, wider application. Its advantage over routine electrophoresis is particularly its speed of analysis.

4. STORAGE CONDITIONS AND QUALITY CONTROL

The stability of LD activity depends on both the storage temperature and the composition of the medium [81–83]. Moreover, the technique used for freezing the samples significantly affects LD activity [84,85], and freezing and thawing may adversely affect LD isoenzymes [18,86]. Some pathological sera could be stored longer than others. This greater stability of some sera may be due to their excessive content of NAD or reduced glutathione level [84]. Individual serum samples vary considerably in their total LD activity and susceptibility to isoenzyme losses during storage. Fig. 6 shows two typical changes in LD isoenzyme profile at 4 °C during a one-week period; in one of the samples the LD pattern is relatively stable whereas in the other it changes rapidly.

The storage of frozen serum samples may result in even more radical isoenzyme pattern alterations, with diminution of the proportion of slow-moving LD isoenzymes and augmentation of the fast-moving fractions [81]. Such alterations may be a possible cause of falsely elevated LD-1/LD-2 ratios. In order to avoid such errors, it is advisable to use samples on the same day as the blood is drawn. If this is not possible, the serum may be stored for 24 h at room temperature. However,

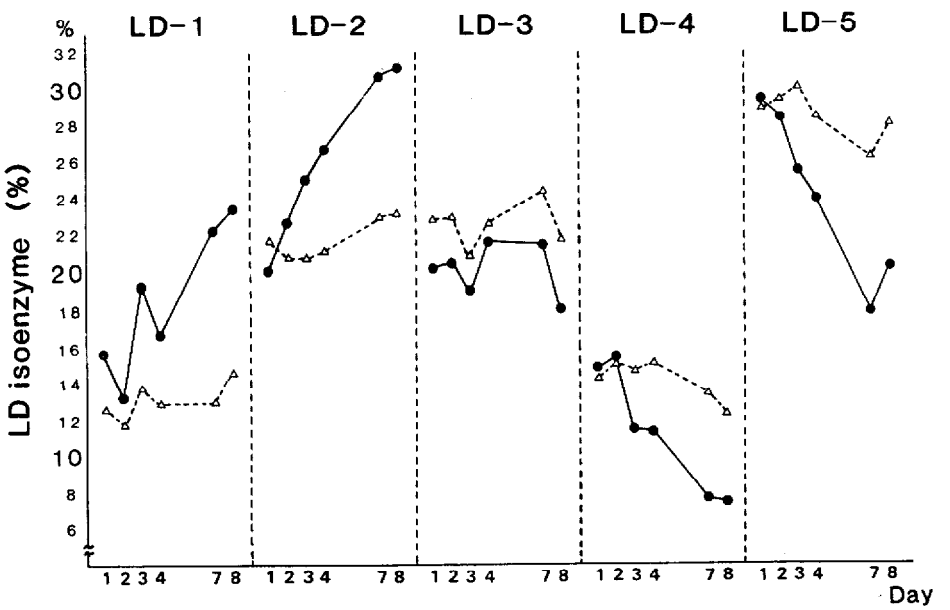

Fig. 6. Stability of LD isoenzymes in two samples at 4 °C. On the first day after sampling the two samples showed similar LD activities and isoenzyme profiles, but differed considerably after three days. The individual samples are designated with solid dots (the relatively unstable sample) and open triangles (the stable sample).

the best storage temperature for retention of LD isoenzyme activity in serum or heparinized plasma specimens is below −60°C.

It is clear that quality control should be applied to LD isoenzyme assays, as to many other laboratory methods. Routinely the reliability should be assessed by daily analysis of quality control sera. McKenzie and Henderson [87] examined the LD isoenzyme contents of 31 commercially available quality control sera and indicated that only pure human quality control sera without LD isoenzyme additions were available for the introduction of a quality control programme for electrophoretic LD isoenzyme assays. The best type of material to use for the quality control of human LD isoenzyme separation would appear to be a serum containing the majority of its activity in the fast-moving LD isoenzymes, having the same mobility and distribution of activity as the fresh normal human serum, and showing five symmetrical peaks of five LD isoenzymes.

We ordinarily include one quality control serum on each cellulose acetate membrane, and check each electrophoretic separation and quantification process. Our procedure is to open a new vial of a commercially available quality control serum at the beginning of the week and use it until the Friday, then discard it. The reconstituted serum is maintained at 4°C throughout the week. We routinely keep Levy–Jennings plots [88] for each LD isoenzyme. The control limit for each LD isoenzyme of the quality control serum is the target average plus or minus twice the usual standard deviation. In this manner we control the analytical conditions, such as changes in electrophoretic buffer, staining solution or quality control serum.

5. CLINICAL APPLICATIONS

The quantitative distribution of the five LD isoenzymes is different and characteristic for each isoenzyme. Therefore, when the LD isoenzymes are released from tissue to serum, as on cell injury, the LD isoenzyme pattern of the serum changes and in a sense resembling the profile of the affected tissue. Tables 3 and

TABLE 3

NORMAL ISOENZYME DISTRIBUTION

Material	Activity (U/l)	Isoenzyme distribution (%)*				
		LD-1	LD-2	LD-3	LD-4	LD-5
Serum**	170-340	25 (18-31)	35 (31-40)	25 (20-29)	8 (5-12)	7 (3-11)
CSF [194]	25.9	53 (38-65)	34 (26-46)	10 (0-24)	2 (0-10)	1 (0-7)
Urine [196]	6.8	62 (35-80)	30 (20-55)	6 (0-20)	2 (0-10)	0 (0)

*Ranges in parentheses.

**Data obtained in our laboratory.

TABLE 4

TISSUE DIFFERENCES OF LD ISOENZYME PROFILES

Organ	Isoenzyme distribution (%)				
	LD-1	LD-2	LD-3	LD-4	LD-5
Heart	57	32	6	3	2
Red blood cells	38	35	25	2	0
Brain	28	32	19	16	5
Kidney	46	33	14	4	3
Liver	0	1	5	9	85
Skeletal muscle	2	7	24	25	42
Skin	0	1	4	15	80
Lung	2	5	19	31	43
Pancreas	10	23	37	13	17
Spleen	8	22	37	26	7
Intestine	10	29	41	14	6
Lymph node	6	22	36	20	16

4 show the normal isoenzyme distribution and LD isoenzyme profiles in different tissues.

5.1. Acute myocardial infarction

Determination of the serum LD isoenzymes in combination with CK or other biochemical markers has become a well established laboratory procedure for helping in the diagnosis of AMI. Cardiac muscle contains mainly LD-1 and LD-2, with a preponderance of LD-1 [89,90]. Hence the release of myocardial LD isoenzymes to normal serum will increase both LD-1 and LD-2 activities. Finally, LD-1 will exceed LD-2 activity [89,91] ("flipped LD" pattern) [90]. LD-1 increases within 12–24 h of the onset of AMI [71,91], as quickly as CK-MB [73], becoming increased before the total LD activity reaches a peak at 48 h [26,92]. By 48 h after AMI, most patients will have a "flipped LD" pattern in their serum [93]. If 24–48 h pass before the patient is examined, the transient rise and fall of CK and CK-MB activities may be missed owing to the short half-lives of those enzymes. However, LD-1 levels may still be abnormal because of its longer half-life [94].

There have been numerous reports on the characteristic changes in the LD isoenzyme pattern (increase in the LD-1/LD-2 ratio) [27,29,30,90,95]. The flipped LD pattern (LD-1 > LD-2) is not as sensitive for MI as CK-MB; however, false positives rarely occur, making the test very reliable [92]. The test may be increased in sensitivity and slightly less in specificity for AMI if the results are interpreted as abnormal when the ratio of LD-1 to LD-2 is 0.76 (or more) [15,31]. On the other hand, some workers propose that the quantitation of serum LD-1 activity alone is diagnostically more reliable [57,58,60,72,73,96], or that the LD-1/total LD ratio has a higher diagnostic value than assaying LD-1 alone [71,74],

or that absolute values of the individual activities can be useful [97]. The LD-1/LD-2 ratio is less person-specific than LD-1 and is probably a more useful diagnostic index for AMI than LD-1 alone [98].

These LD isoenzyme patterns have also been employed successfully to monitor heart injury after coronary bypass and other cardiac surgery [91,99,100]. LD-1 and LD-2 levels are increased in myocarditis [92] and unstable angina [101]. Pulmonary embolism and oesophageal injury may mimic AMI in their clinical presentation. In these patients, AMI can be distinguished by assaying their LD isoenzyme pattern [92,102].

Patients with AMI, left-heart failure and passive congestion of the liver will show increases not only in LD-1 but, commonly, also in LD-5 [91,94]. The LD activity or the LD-1/LD-2 ratio observed at the peak of CK activity is a good indicator of survival [104], while an abnormal LD-5 level is a predictor of early mortality after infarction [105]. Most clinical laboratories can use an immunochemical method as a screening test or urgent test and an electrophoretic method or chromatographic method as an additional diagnostic test.

5.2. Skeletal muscle diseases

The LD isoenzyme pattern of most normal skeletal muscle shows a predominance of LD-5 and the serum LD-5 activity increases in acute rhabdomyolysis [106]. However, an increase in fast-moving LD isoenzymes has been seen in sera of patients with muscular dystrophy [106–108]. Wieme and Herpol [108] demonstrated a reduction in the proportion of LD-5 in human dystrophic muscle. This observation has since been confirmed not only in dystrophic muscle but also in a wide variety of neuromuscular diseases [109,110]. The rapid elimination rate of the slow-moving isoenzymes from the circulation may be the reason for the changes in the isoenzyme distribution (predominance of fast-moving isoenzymes) in serum.

5.3. Liver diseases

The serum LD-5 level is a very sensitive indicator of hepatocellular damage [28,111,112] and elevations of LD-5 are commonly seen in hepatitis [6,113], liver hypoxia (including a sequel of congestive cardiac failure), liver congestion, drug toxicity, cirrhosis, tumours or trauma [28,112,114–117].

5.4. Haematological diseases

In megaloblastic and haemolytic anaemias the serum LD activity is always almost elevated [118–120] and this elevation is due to an increase in LD-1 and LD-2 [6,118–120]. The destruction of immature and abnormal erythroid precursors in the bone marrow is considered to be the most likely source of the raised serum LD activity and fast-moving isoenzymes in megaloblastic anaemia [119]. In iron deficiency anaemia, pancytopaenia and secondary polycythaemia the LD isoenzyme pattern is reportedly almost normal [118].

5.5. Malignancy

Most reports emphasize the increase in M subunits in tumours of all origins [122] and the shift toward the slow-moving isoenzymes in many human malignant neoplasms [123,124]. On the other hand, Schapira et al. [125] found a shift toward the fast-moving isoenzymes, LD-1 and LD-2, in human rhabdomyosarcoma, which was interpreted as a deviation to the foetal isoenzyme pattern.

Concerning the LD isoenzyme pattern in malignant tumour tissues, numerous studies have been carried out over the past three decades. Three different types of LD isoenzyme patterns have been observed in a variety of malignancies. An elevated slow-moving LD has been noted in malignant prostatic tumours [126,127], lung tumours [128], uterine tumours [129], gastric cancer [130], colonic cancer [131], breast cancer [132], bladder cancer [133] and several types of brain tumours [134]. A general increase in LD-2, LD-3 and LD-4 has been observed in the serum of patients with leukaemia [135–137], malignant lymphoma [135,138], neuroblastoma [139], pheochromocytoma [140], oral cancers [141], bronchial cancer [142] and several types of brain tumours [134]. Up to the present time, serum LD-1 has been found elevated in the serum of patients with several types of brain tumours [134] and various types of germ-cell tumours of the ovaries, testis and mediastinum [135,143–145]. Several types of tumours produced an unusual extra band of LD isoenzymes in serum and tumour tissue of brain tumour [146,147], oesophageal cancer [148] and neuroblastoma [149].

LD isoenzymes may serve as a useful tumour marker not only for diagnosis but also for post-therapy surveillance in patients with malignancy. LD isoenzymes have been successfully used as indicators for prognosis during therapy. A return of the serum LD isoenzymes to normal values, following treatment, was found to correlate with a favourable patient response to therapy [124,126,130–132,134–138,141–145].

5.6. Miscellaneous diseases

A pathological elevation of fast-moving isoenzymes in serum is a sign of parenchymatous brain damage [150] or renal infarction if the possibility of myocardial infarction or in vivo haemolysis is eliminated. In addition, LD isoenzymes in the following diseases have been reported to be associated with clinical investigation: diabetes mellitus [151], rheumatoid arthritis [152], lupus nephritis [153], collagen disease [154], measles infection [155], colonic infarction [156] and pulmonary infarction [6].

6. ABNORMAL PATTERNS INCLUDING ENZYME-IMMUNOGLOBULIN COMPLEX

Kreutzer [157] found that LD isoenzymes of several patients showed abnormal electrophoretic mobilities. Ganrot [158] attributed this abnormal mobility to the binding of LD isoenzymes to immunoglobulin A. The LD–immunoglobulin complex is, indeed, a common cause of abnormal electrophoretic patterns. The abnormal electrophoretic pattern may result from the presence of an extra LD

fraction, changed mobility, a change in the molecular structure (and/or size) of a fraction or from the distortion of one or more regularly present bands [158–161]. The heterogeneity of the patterns reflects the binding affinities of immunoglobulins for LD isoenzymes [160]. Occasionally, immunoglobulins interfere with enzyme activity even without any accompanying abnormal electrophoretic mobility [162,163]. There was a case report in which the LD–immunoglobulin G-complex produced both an abnormal LD isoenzyme pattern and a loss of enzyme activity [164].

Baxi et al. [165] showed a possible relationship between surface antigen of hepatitis B virus and LD isoenzyme, appearing as an anomalous band between LD-4 and LD-5. Vyas et al. [166] designated the anomalous band as LD-5ex. They reported that the LD-5ex was associated with the e antigen of hepatitis B virus and might provide a simple, sensitive and rapid marker for detecting this antigen [166].

Cabello et al. [167] described some patients having an additional band on the cathodic side of LD-5. They called the extra band LD-6 and noted that all of the patients had arteriosclerotic cardiovascular disease and episodes of shock, and most died during hospitalization. Podlasek et al. [168] found that LD-6 did not require lactate as substrate and could be enhanced by ethanol added to the substrate. The characteristics are similar to alcohol dehydrogenase activity [169]. In clinical applications, LD-6 may be a useful marker for severe liver injury and an indicator showing poor prognosis [167–169].

7. GENETIC VARIANTS

Boyer and Fainer [170] found a deviating electrophoretic pattern in a Nigerian patient. This altered pattern was apparently due to an H subunit variant, as there were five components in the LD-1 position, four in the LD-2 position and three in the LD-3 position. Nance et al. [171] described an M subunit variant with a faster anodal mobility in four individuals in a Brazilian family. Later, more electrophoretic variants of LD have been identified in human populations [172–174]. All these variants can be grouped into slow and fast types according to their relative rates of electrophoretic mobility.

Among the Guaymi Indians of Panama and Costa Rica an electrophoretic variant of LD-H(B) subunit, named LD_BGUA-1, has been reported [175,176]. The variant subunits are always enzymatically inactive, while the tetrameric combinations which contain even one active subunit possess enzymatic activity [176]. On the other hand, complete deficiency of LD-H subunit or -M subunit activity has been reported in the Japanese population [177–179]. The prevalence of heterozygous individuals with either of these LD subunit deficiencies was estimated to be about 0.15–0.20% [179]. From the clinical viewpoint, homozygous individuals with M subunit deficiency have episodes of myoglobinuria after strenuous exercise, difficulties in delivery and skin eruptions [178,180,181].

A new form of LD isoenzymes has been reported and designated LD_k [182]. The LD_k form is a highly basic variant, inhibited by physiological concentrations of oxygen. The major interest in this enzyme form is that its activity has been

reported to increase in rat cells infected with the Kirsten murine sarcoma virus and in many human carcinomas [182,183]. Further work [184,185] suggested that LD_k may not be a separate LD type, and its activity corresponds to that of LD-5 measured under special conditions.

A unique electrophoretic form of LD has been reported and designated LD-Z [186]. This isoenzyme form is observed in the electrophoretic profile as slightly cathodal to LD-2 and occurs in early placenta, hydatidiform mole, human choriocarcinoma and several types of choriocarcinoma cell line, and may be indicative of the trophoblastic origin of the cells [186,187].

8. LD-X (LD-C)

Blanco and Zinkham [188] first identified LD-C_4, an isoenzyme confined to the testes and sperm, and showed that this isoenzyme does not disturb the harmony of the five isoenzyme system of LD in serum. The isoenzyme is present only in primary spermatocytes and in later stages of the germinal cell line; its activity in seminal plasma has been regarded as a sign of leakage from the spermatozoa or from their precursor cells. The amount of LD-C_4 was related to testicular maturity and some forms of testicular dysfunction were associated with the disappearance of LD-C_4 [189,190]. Thus, the quantitative analysis of LD-C_4 activity in seminal plasma has been developed [190,191]. Immunization with LD-C_4 suppresses fertility [192].

9. BIOLOGICAL FLUIDS (EXCEPT SERUM)

9.1. Cerebrospinal fluid

The LD activity in cerebrospinal fluid (CSF) is elevated in central nervous systems affected by cancer, infectious disease, trauma or cerebrovascular accidents [193,194]. The LD isoenzyme pattern in CSF may be of potential value in assessing anoxic brain damage [195], in helping to differentiate tubercular or viral meningitis from pyogenic meningitis [193,194,196], in detecting leptomeningeal infiltration by systemic cancer [197] or in following the course of the illness [197]. Controls reveal predominance of LD-1 and LD-2 [193–196] (see Table 3). Increases in LD-3, LD-4 and LD-5 are evident in meningitis with intense increases in LD-3 in tubercular meningitis and LD-5 in pyogenic meningitis [196].

9.2. Urine

To determine the localization of urinary tract infections, the LD isoenzyme pattern in urine, especially LD-5, has been studied [198,199]. The usual isoenzyme pattern was of LD-1 and LD-2 predominating [198,199] (see Table 3). The concentration of LD-5 in pyelonephritis is significantly higher than in bladder infections [199–201]. The LD-5 in urine is derived either from renal cortical

tissues damaged by pyelonephritis or acute tubular necrosis [202] or from leukocytes or erythrocytes [198].

9.3. Effusions

The increase in slow-moving LD isoenzymes in malignant effusions can be used for the differentiation of benign effusions from malignant exudates [103]. In pleural fluid an increase in LD-5 is a good marker of malignant pleural effusion. It is probably due to the leakage of LD-5 from the pleural malignant cells directly in the pleural fluid. In contrast, the increase in LD-5 in pleural fluid during congestive heart failure may arise from serum (transudative pleural effusion) and emanate from liver impairment. The comparison of LD isoenzyme patterns between serum and pleural fluid is a valuable tool in the diagnosis of pleural effusions [121].

10. SUMMARY

The analytical procedures for LD isoenzymes include electrophoresis, chromatography, immunochemical and kinetic methods. Electrophoretic methods are generally preferred because the resulting patterns are directly observable and all five isoenzymes are resolved in a single procedure. Chromatographic methods, with the introduction of HPLC, have recently been perfected in terms of speed, resolution, precision and accuracy. Immunochemical methods and kinetic methods are attractive because of their speed and simplicity. Therefore, the latter methods are used mainly for assaying acute myocardial infarction, where generally the determination of LD-1 and LD-2 is sufficient. In all other instances, however, electrophoretic separation is currently preferred. Ion-exchange high-performance procedures are useful prospects, particularly in view of their velocity in comparison with electrophoresis. In general, the LD isoenzymes assay contributes considerably to diagnosis, but the results must be used with an adequate knowledge of biochemistry, physiology and the advantages and drawbacks of the different assay methods used.

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