Lactic Dehydrogenase

Hans-Ulrich Bergmeyer, Erich Bernt and Benno Hess

Lactic dehydrogenase (LDH), the "reduzierende Gärungsferment" (*Warburg*[1]), was first crystallized from rat muscle in 1943[2]. The best known lactic dehydrogenases are the enzymes from skeletal[3,4] and heart muscle[5]. Lactic dehydrogenases from bacteria[6] and yeast[7] are not linked to the pyridine nucleotide coenzymes.

In human organs the LDH activity decreases in the following order[8] (related to g. fresh weight): kidney > heart > skeletal muscle > pancreas > spleen > liver > lung > serum.

The activity of LDH of animal origin is measured spectrophotometrically[2] (see equation 1). Measurements of the activity by the colour reactions (*e.g.*[9]) of the 2,4-dinitrophenylhydrazone of the unreacted pyruvate are difficult, because the DPNH produced in the reaction also forms a hydrazone which absorbs in the same region[10].

The lactic dehydrogenase activity of serum is composed of several enzyme proteins with the same action and substrate specificity but of different origin. The lactic dehydrogenases in serum which originate from liver, heart, skeletal muscle, erythrocytes, tumours, *etc.* are not only different from one another, but themselves consist of several enzymatically active fractions which can be separated from each other[11–27]. Such enzymes, which differ in their protein structure and therefore in the

---

1) *O. Warburg:* Wasserstoffübertragende Fermente. Dr. W. Saenger, Berlin 1948, p. 40.
2) *F. Kubowitz* and *P. Ott*, Biochem. Z. *314*, 94 [1943].
3) Cf. *A. Kornberg* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 441.
4) *G. Beisenherz, J. H. Boltze, T. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].
5) Cf. *J. B. Neilands* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 449.
6) *J. Szulmajster, M. Grunberg-Manago* and *C. Delavier-Klutekko*, Bull. Soc. Chim. biol. *35*, 1381 [1953].
7) Cf. *M. Dixon* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 444.
8) *F. Wróblewski*, Scand. J. Clin. & Lab. Invest. *10*, 230, Suppl 31; Int. Congr. clin. Chem. Stockholm 1957.
9) *P. G. Cabaud, F. Wróblewski* and *V. Ruggiero*, Amer. J. clin. Pathol. *30*, 234 [1958].
10) *H.-U. Bergmeyer* and *E. Bernt*, unpublished.
11) *Th. Wieland* and *G. Pfleiderer* Biochem. Z. *329*, 112 [1957].
12) *G. Pfleiderer* and *D. Jeckel*, Biochem. Z. *329*, 370 [1957].
13) *Th. Wieland, G. Pfleiderer*, and *F. Ortanderl*, Biochem. Z. *331*, 103 [1959].
14) *Th. Wieland, G. Pfleiderer, I. Haupt* and *W. Wörner*, Biochem. Z. *332*, 1 [1959].
15) *B. Hess*, Klin. Wschr. *36*, 985 [1958].
16) *B. Hess*, Ann. N. Y. Acad. Sci. *75*, 292 [1958].
17) *B. Hess* in *W. H. Hauss* and *H. Losse:* Struktur und Stoffwechsel des Herzmuskels. G. Thieme, Stuttgart 1959, p. 128.
18) *B. Hess* and *S.-I. Walter*, Klin. Wschr. *38*, 1080 [1960].
19) *E. S. Vesell* and *A. G. Bearn*, Proc. Soc. exp. Biol. Med. *94*, 96 [1957].
20) *E. S. Vesell* and *A. G. Bearn*, J. clin. Invest. *37*, 672 [1958].
21) *E. S. Vesell* and *A. G. Bearn*, Ann. N. Y. Acad. Sci. *75*, 286 [1958].
22) *B. R. Hill*, Cancer Res. *16*, 460 [1956].
23) *F. W. Sayre* and *B. R. Hill*, Proc. Soc. expl. Biol. Med. *96*, 695 [1957].
24) *B. R. Hill*, Ann. N. Y. Acad. Sci. *75*, 304 [1958].
25) *R. J. Wieme* and *L. Demeulenaere*, Acta gastro-ent. belg. *22*, 69 [1959].
26) *N. O. Kaplan, M. M. Ciotti, M. Hamolsky* and *R. E. Bieber*, Science [Washington] *131*, 392 [1960].
27) *R. Richterich, E. Gautier, W. Egli, K. Zuppinger* and *E. Rossi*, Klin. Wschr. *39*, 346 [1961].

optimum conditions for their action, but have the same specificity (in this case towards L-lactate), are termed "isozymes"[28] or "isoenzymes"[29].

A method is described below which gives approximately optimum conditions for the measurement in serum of the LDH from heart and liver (Fig. 1).

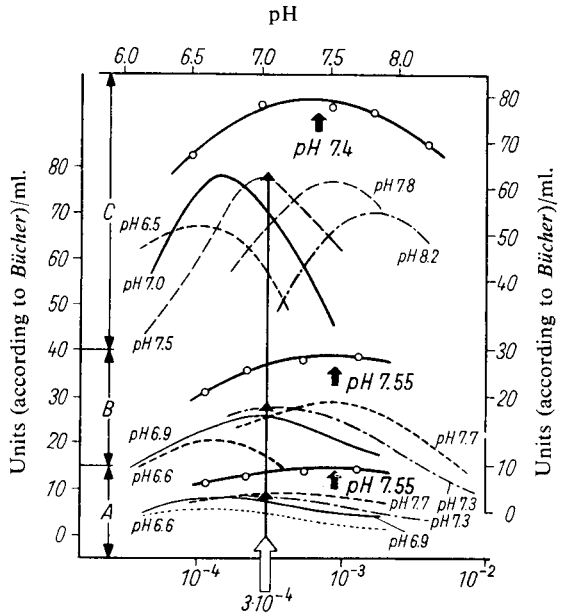

Fig. 1. Determination of the optimum conditions for the measurement of LDH activity in serum. 25°C; 0.05 M phosphate buffer

Section A: Normal serum
Section B: Serum in hepatitis
Section C: Serum after myocardial infarction
Left hand and bottom ordinates (broken and thin-lined curves): Dependence of the activity on the pyruvate concentration with constant pH (which is given on each curve)

Right hand and upper ordinates (thick-lined curves —o—o—): Dependence of the activity on the pH with constant pyruvate (3×10^{-4} M) (optimum activity)

Principle

Lactic dehydrogenase (LDH) catalyses the reaction:

(1) $$\text{Lactate} + \text{DPN}^+ \rightleftharpoons \text{pyruvate} + \text{DPNH} + \text{H}^+$$

The equilibrium is far on the side of lactate and diphosphopyridine nucleotide (DPN). The LDH activity is measured by the rate of consumption of pyruvate and reduced diphosphopyridine nucleotide (DPNH); the decrease of optical density at 340 or 366 mμ due to the oxidation of DPNH is measured.

---

[28] *C. L. Markert* and *F. Møller*, Proc. nat. Acad. Sci. USA *45*, 753 [1959].
[29] *F. Wróblewski* and *K. Gregory*: Proc. 14th Internat. Congr. clin. Chem. Edinburgh, 1960, E. & S. Livingstone Ltd., Edinburgh and London 1961, p. 62.

Optimum Conditions for Measurements

The most important characteristics for the assay of activity of the individual lactic dehydrogenases are their different substrate and pH optima. Measurements which are not carried out under optimum conditions naturally result in values for the activity which are too low. The optimum conditions for the measurement of the enzyme in serum after myocardial infarction, in liver damage, blood diseases and tumours have been more or less established[15-24,30,31]. The measurements shown in Fig. 1 were made with phosphate buffer at 25°C[32]. The optima of activity with variation of the pyruvate concentration are strongly dependent on pH. A pyruvate concentration of 3×10^{-4} M at pH 7.5 gives nearly optimum activities in human serum for the enzymes from liver and heart. The temperature of the measurements is 25°C and the DPNH concentration is 1.3×10^{-4} M. The dependence of the LDH activity on the DPNH concentration is not shown in Fig. 1. DPNH over the range of about 5×10^{-5} to 5×10^{-4} M gives a wide activity optimum. With higher temperatures, for example, 37°C, higher substrate concentrations are required for optimum activity.

Reagents*)

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Dipotassium hydrogen phosphate, K_2HPO_4, A. R.
3. Sodium pyruvate
   commercial preparation, see p. 1027.
4. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.

Preparation of Solutions

I. Phosphate-pyruvate solution (0.05 M phosphate buffer pH 7.5; 3.1×10^{-4} M pyruvate):
Dissolve 700 mg. K_2HPO_4, 90 mg. KH_2PO_4 and 3 mg. Na pyruvate in doubly distilled water and make up to 80 ml.

II. Reduced diphosphopyridine nucleotide (*ca.* 8×10^{-3} M β-DPNH):
Dissolve 10 mg. DPNH-Na_2 in 1.5 ml. phosphate-pyruvate solution (I).

Stability of the solutions

Store all the solutions, stoppered, in a refrigerator at 0–4°C. Prepare the DPNH solution freshly each week. Deterioration of the buffer is usually due to bacterial contamination, which can be prevented by addition of a few drops of chloroform.

Procedure

Use only fresh serum free from haemolysis.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3.0 ml.; temperature 25°C (preferably use a constant temperature cuvette holder).

A control cuvette is not necessary. Before the assay bring the reaction mixture and the serum to 25°C (water bath).

---

*) Complete reagent kits are available commercially, see p. 1036.

30) *R. E. Thiers* and *B. Vallee*, Ann. N. Y. Acad. Sci. *75*, 214 [1957].
31) *E. Schmidt* and *F. W. Schmidt*, personal communication.
32) *H.-U. Bergmeyer*, Lecture, Kongr. f. Lab. Med., Berlin April 1961.

Pipette successively into the cuvette:

2.85 ml. phosphate-pyruvate solution (I)
0.05 ml. DPNH solution (II)
0.10 ml. serum.

Mix, immediately start a stopwatch and read the optical density at minute intervals for 3 to 5 min.

The measured optical density difference ΔE/min. should not be greater than 0.020/min.; otherwise dilute the serum 1:10 with the phosphate-pyruvate solution (I).

Calculations

According to *Wróblewski* and *La Due*[33] a unit is the amount of LDH which changes the optical density of DPNH at 340 mμ by 0.001 in 1 min., in a 3 ml. assay mixture and at 24–27° C.

It therefore follows that with 0.1 ml. of serum

(2) $(\Delta E_{340}/\text{min.}) \times 10000 = \text{LDH units/ml. serum}$

(3) $(\Delta E_{366}/\text{min.}) \times 18900 = \text{LDH units/ml. serum}$

Obtain the mean of the measured ΔE/min. values and use these for the calculations. Depending on the type of serum the reaction curves are either linear or non-linear. In the latter case (the values for ΔE/min. decrease with time) use the mean of the first three readings.

Normal values

See page 705.

Example

0.1 ml. of normal serum was analysed and the following optical densities were measured at 366 mμ (linear curve):

0 min.	0.402	
		ΔE = 0.015
1 min.	0.387	
		ΔE = 0.014
2 min.	0.373	
		ΔE = 0.016
3 min.	0.357	
		ΔE = 0.015
4 min.	0.342	
	Mean	ΔE/min. = 0.015

$0.015 \times 18900 = 284$ units/ml. serum (according to *Wróblewski*)

Conversion to other units

1. For DPN-linked dehydrogenases a unit according to *Bücher et al.*[4] is the amount of enzyme contained in 1 ml. which changes the optical density of DPNH at 366 mμ by 0.100 in 100 sec. at 25° C and with a 1 cm. light path.

Therefore at 25° C:

1 unit (*Bücher*): ΔE_{366}/100 sec. = 0.100
ΔE_{366}/min. = 0.060
ΔE_{340}/min. = 0.113
for a 3 ml. assay mixture ΔE_{340}/min. = 0.0377

1 unit (*Wróblewski*): ΔE_{340}/min. = 0.001

37.7 units (*Wróblewski*) = 1 unit (*Bücher*)
0.0265 units (*Bücher*) = 1 unit (*Wróblewski*)

[33] *F. Wróblewski* and *J. S. LaDue*, Proc. Soc. exp. Biol. Med. *90*, 210 [1955].

2. According to *Racker et al.*[34] a unit is the amount of enzyme which converts 1 μmole of substrate/min. at 25° C.

Therefore:

1 unit (*Racker*): 1 μmole/min.

1 unit (*Wróblewski*): ΔE_{340}/min. = 0.001 (3 ml. assay mixture)

as ΔE_{340}/min. = 0.001 corresponds to the conversion of 4.82×10^{-4} μmoles of substrate/3 ml.

1 unit (*Wróblewski*) = 4.82×10^{-4} units (*Racker*)

or

1 unit (*Racker*) = 2073 units (*Wróblewski*)

To calculate directly from the measured values with 1 ml. serum:

Measurements at 340 mμ:

(2)	(ΔE/min.) × 10000	= units (*Wróblewski*)
(2a)	(ΔE/min.) × 265	= units (*Bücher*)
(2b)	(ΔE/min. × 4.82	= units (*Racker*)

Measurements at 366 mμ:

(3)	(ΔE/min.) × 18900	= units (*Wróblewski*)
(3a)	(ΔE/min.) × 501	= units (*Bücher*)
(3b)	(ΔE/min.) × 9.1	= units (*Racker*)

Sources of Error

Experience has shown that none of the substances present in blood interfere in the assay. The concentration of pyruvate in normal human serum is about 2 orders of magnitude lower than that of the assay mixture and therefore, contrary to several reports in the literature, it cannot affect the optimum concentration.

It is necessary to determine the optimum concentration of pyruvate (that of DPNH is less important) for serum LDH from other organs. For example, in carcinoma of the bronchus it is 2×10^{-3} M[35].

Stability of the Enzyme in the Serum Sample

The loss of activity after storage of serum at different temperatures has been measured by *Südhof et al.*[36]. Storage at 4° C or in the frozen state results in *ca.* 15% loss of activity in 12 hours and 27% in 24 hours. Similar values are obtained at room temperature.

Details for Measurements in Tissues and Other Body Fluids

LDH is a cytoplasmic enzyme[37]; simple homogenization in a *Potter-Elvehjem* homogenizer (refer to p. 49) is sufficient to completely extract the enzyme. The activity is determined in the supernatant after centrifuging the homogenate at high speed. For measurements on, for example, liver punctures, 10 mg. fresh weight of tissue is sufficient[38].

---

[34] *J. Cooper, P. A. Srere, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].
[35] *E. Schmidt* and *F. W. Schmidt*, personal communication.
[36] *H. Südhof* and *E. Wötzel*, Klin. Wschr. *38*, 1165 [1960].
[37] *Th. Bücher* and *P. Baum*, Lecture, Dtsch. Kongr. f. ärztl. Fortbildung, Berlin 1958.
[38] *E. Schmidt, F. W. Schmidt* and *E. Wildhirt*, Klin. Wschr. *36*, 172 [1958].

The optimum conditions for human serum given in Fig. 1 do not necessarily hold for sera from other species or for other organs and body fluids. The following concentrations are suitable for measurements on cerebrospinal fluid[39]: 8×10^{-4} to 2×10^{-2} M DPNH; 3×10^{-4} to 1×10^{-3} M pyruvate; pH 6.5 to 7.4 (25° C).

Differentiation of the Lactic Dehydrogenases in Serum Originating from Specific Organs

The individual types of LDH proteins can be separated from each other electrophoretically[11–14] or chromatographically[15–18,40,41]. Liver and skeletal muscle LDH occurring in serum can be determined separately in the presence of LDH from heart, kidney and erythrocytes.

Principle

The LDH activity of a dialysed serum sample is assayed before and after treatment with DEAE-cellulose. Under defined conditions, the DEAE-cellulose adsorbs the heart muscle-type of LDH protein, while the liver-type of LDH protein remains in solution*). For the principle of the spectrophotometric assay, see p. 737.

Reagents

Additional to 1—4 on page 738:

5. Sodium dihydrogen phosphate, $NaH_2PO_4\cdot H_2O$, A. R.
6. Disodium hydrogen phosphate, $Na_2HPO_4\cdot 12\ H_2O$, A. R.
7. Sodium chloride, A. R.
8. Diethylaminoethyl-cellulose, DEAE-cellulose
   from Serva, Heidelberg, Germany**). Capacity: 0.6 mequiv./g.

Preparation of Solutions

Additional to I and II on page 738:

III. Phosphate buffer (2×10^{-2} M; pH 6.0):
Mix 12.3 ml. of a solution of 7.164 g. $Na_2HPO_4\cdot 12\ H_2O$/100 ml. doubly distilled water with 87.7 ml. of a solution of 2.76 g. $NaH_2PO_4\cdot H_2O$/100 ml. doubly distilled water and dilute to 1000 ml. with doubly distilled water.

IV. DEAE-cellulose suspension (*ca.* 10% w/v):
Suspend 5 g. DEAE-cellulose in 50 ml. phosphate buffer (solution III), disperse thoroughly, allow to sediment for *ca.* 45 min. and decant from the coarse, sedimented material. Repeat the sedimentation procedure three times. Wash the fine, similar-sized adsorbent

*) The method is also suitable for the differentiation of other enzymes and proteins apart from the LDH isoenzymes, providing that they are specifically adsorbed by DEAE-cellulose. For example, serum free from α-globulin can be produced by this method.

**) Or a complete reagent kit (see p. 1036) from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Other preparations have not been tried.

39) *H. R. Tyler* and *L. Bromberger*, J. nerv. ment. Dis. *130*, 54 [1960].
40) *B. Hess* and *S.-I. Walter*, Klin. Wschr. *39*, 213 [1961].
41) *B. Hess* and *S.-I. Walter*, Ann. N. Y. Acad. Sci. *94*, 890 [1961].

particles with phosphate buffer (solution III) until the pH of the washings is constant (pH 6.0) and then resuspend in 50 ml. phosphate buffer (solution III). Add a few drops of toluene to prevent bacterial contamination and store at 0—4° C.

V. Phosphate-NaCl solution ($2 \cdot 10^{-1}$ M phosphate, 2×10^{-1} M NaCl; pH 6.0):
Mix 12.3 ml. of a solution of 7.164 g. $Na_2HPO_4 \cdot 12\ H_2O$/100 ml. doubly distilled water with 87.7 ml. of a solution of 2.76 g. $NaH_2PO_4 \cdot H_2O$/100 ml. doubly distilled water. Dissolve 1.169 g. NaCl in this solution and make up to 100 ml.

Procedure

Preliminary treatment of the experimental material

Dialyse 3 ml. of serum or tissue extract for 2 hours against 1000 ml. phosphate buffer (solution III). A small amount of protein precipitates out on dialysis (equal amounts of α- and β-globulins), but no LDH activity is lost. Use the contents of the dialysis sac for the fractionation without filtering.

Fractionation on DEAE-cellulose

In principle, it is sufficient to adsorb the heart muscle-type LDH on the DEAE-cellulose and then to assay the activity in the supernatant after removal of the cellulose. If it is wished to check the method, the adsorbed LDH is eluted and the LDH activity of the eluate is also measured.

Adsorption:

Suspend the DEAE-cellulose evenly by shaking. Use pipettes with a wide tip (or broken tip).

Pipette into a conical centrifuge tube:

2 ml. dialysed serum
2 ml. DEAE-cellulose suspension (IV).

Mix with a thin glass rod, allow to stand for 10 min. with occasional stirring. Centrifuge at *ca.* 4000 g for 5—10 min. and pour off the supernatant into a dry test tube.

Elution:

Add to the residue in the centrifuge tube

ca. 3 ml. phosphate buffer (solution III),

mix and centrifuge. Discard the supernatant. Repeat the washing 2—3 times. This removes any of the liver-type LDH activity which may have been carried down with the cellulose.

Add sufficient

phosphate buffer-NaCl solution (V)

to the sediment to make up to 4 ml., stir thoroughly and centrifuge for 3 min. at 3000 g. Pour the supernatant (eluate) into a dry test tube.

To check the method by preparing a balance of the activity (but without obtaining the exact fraction of LDH adsorbed), the washing procedure can be omitted and the adsorbed LDH + any liver-type LDH carried down with the cellulose sediment can be eluted immediately with solution V.

Activity measurements

As described above (p. 738).

Calculations

Use the formula on p. 739 to calculate the activity in the dialysed serum, in the supernatant after adsorption and in the eluate. The activity is obtained in units/ml. of solution taken for the assay. As 1 g. DEAE-cellulose binds 3 ml. of water, the "solute space" with 200 mg. cellulose in a final volume of 4 ml. is 4.0 − 0.6 = 3.4 ml.

The following formula gives the fraction of the LDH activity which is not adsorbed as a percentage of the total activity in the sample taken:

$$(4) \qquad \%\ \mathrm{LDH_{liver\text{-}type}} = 100 \times \frac{3.4\ A_2}{2\ A_1} = 100 \times \frac{1.7\ A_2}{A_1}$$

and the percentage of LDH adsorbed is

$$(5) \qquad \%\ \mathrm{LDH_{heart\ muscle\text{-}type}} = 100 \times \left(1 - \frac{1.7\ A_2}{A_1}\right)$$

where A_1 = activity/ml. dialysed serum
A_2 = activity/ml. supernatant

The result given by equation (5) includes the fraction of the liver-type LDH carried down with the cellulose sediment and the result given by equation (4) is too low by this amount.

The activity of the heart muscle-type LDH can be calculated more exactly by A_3 (activity/ml.) in the eluate of the washed cellulose sediment:

$$(6) \qquad \%\ \mathrm{LDH_{heart\ muscle\text{-}type}} = 100 \times \frac{3.4\ A_3}{2\ A_1} = 100 \times \frac{1.7\ A_3}{A_1}$$

If the values given by equations (4) and (6) are added and compared with 2 A_1 a deficit is obtained. The amount of liver-type LDH carried down on the DEAE-cellulose can be determined by omitting the washings and eluting the cellulose sediment directly. Let the activity of this eluate be A_4, then the total amount of LDH on the cellulose sediment is:

$$(7) \qquad \%\ \mathrm{LDH_{sediment}} = 100 \times \frac{3.4\ A_4}{2\ A_1} = 100 \times \frac{1.7\ A_4}{A_1}$$

The difference between the values given by equations (7) and (6) is the percentage of liver-type LDH activity carried down by the cellulose. The sum of the fraction of LDH activity carried down with the cellulose, the liver-type LDH (according to equation (4)) and the heart muscle-type LDH (according to equation (6)) should be a 100. Any deviation gives the error of the method.

Sources of Error

1 g. DEAE-cellulose adsorbs about 150 mg. protein[42], the exact amount depending on the capacity of the preparation. Therefore the cellulose suspension should be prepared according to the capacity. The 0.12 mequiv. used in the assay should not be completely utilized. According to[40] 1 g. cellulose adsorbs about 250 LDH units*). A sufficiently sharp separation of the fractions is only obtained if the conditions of the method are strictly adhered to.

*) Defined according to[34].

[42] *H. A. Sober* and *A. E. Peterson*, J. Amer. chem. Soc. *76*, 1711 [1954].