L-(+)-Lactate

Determination with Lactic Dehydrogenase and DPN

Hans-Jürgen Hohorst

Principle

Lactic dehydrogenase (LDH) catalyses the oxidation of L-lactate by diphosphopyridine nucleotide (DPN):

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

The equilibrium of the reaction, which lies far to the left, has a constant K_c of 2.9×10^{-12} [moles/l.] (25°C)[1]. The reaction products must be removed from the mixture to obtain quantitative oxidation of L-lactate. Protons are bound by use of an alkaline reaction medium and pyruvate is trapped as the hydrazone. The basic equation for the spectrophotometric assay of L-lactate is:

(2) $$\text{L (+)-Lactate} + \text{DPN}^+ + \text{hydrazine} \xrightarrow[\text{pH 9.5}]{\text{LDH}} \text{pyruvate hydrazone} + \text{DPNH} + \text{H}_3\text{O}^+$$

The equilibrium constant for this reaction[2] is $K_c \approx 7 \times 10^2$ at pH 9.5 and 25°C. Relatively high concentrations of DPN and LDH are necessary to obtain a quantitative and sufficiently fast reaction (see under "Sources of Error"). The course of the reaction is followed spectrophotometrically by the increase in optical density due to the formation of DPNH.

Reagents *)

1. Hydrazine sulphate, A. R.
2. Glycine, A. R.
3. Sodium hydroxide, A. R., 2 N
4. Potassium carbonate, K_2CO_3, A. R.
5. Methyl orange indicator
6. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
7. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (Titriplex III, Trilon B, Versene).
8. Diphosphopyridine nucleotide, DPN
   free acid. Commercial preparation, see p. 1010. Only preparations containing at least 75% β-DPN are suitable.
9. Lactic dehydrogenase, LDH
   crystalline, from skeletal muscle, suspension in 2.1 M ammonium sulphate solution. Commercial preparation, see p. 986.

Purity of the enzyme preparation

The LDH preparation should have a specific activity of at least 15000 units/mg. (according to *Bücher* **) or 270 units/mg. (according to *Racker* **). Contamination by malic dehydrogenase and glycerol-1-phosphate dehydrogenase should not exceed 0.03% (relative to the LDH activity).

*) Complete reagent kits are available commercially, see p. 1035.
**) Definition of unit, see p. 32, 33.

1) *H. H. Hohorst*, Ph. D.-Thesis, Universität Marburg, 1960; according to *N. O. Kaplan et al.*, J. biol. Chemistry *221*, 838 [1956], the value is 1.2×10^{-12}; *E. Racker*, *ibid. 184*, 313 [1950], found $K_c = 4.4 \times 10^{-12}$.

2) *H. J. Hohorst*, Diploma-Thesis, Universität Marburg, 1959.

Preparation of Solutions (for *ca.* 20 determinations).

Prepare all solutions with fresh, doubly distilled water.

I. Potassium carbonate (*ca.* 5 M):
Dissolve *ca.* 69 g. K_2CO_3 in distilled water and make up to 100 ml.

II. Methyl orange indicator:
Dissolve 50 mg. methyl orange in distilled water and make up to 100 ml.

III. Perchloric acid (*ca.* 6% w/v):
Dilute 7.7 ml. $HClO_4$ (sp. gr. 1.67) to 150 ml. with distilled water.

IV. Hydrazine-glycine buffer (0.4 M hydrazine; 1 M glycine; pH 9.5):
Suspend 7.5 g. glycine, 5.2 g. hydrazine sulphate and 0.2 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in a little distilled water, add 51 ml. 2 N NaOH and dilute to 100 ml. with distilled water.

V. Diphosphopyridine nucleotide (*ca.* 5×10^{-2} M β-DPN):
Dissolve 40 mg. DPN in distilled water and make up to 1 ml.

VI. Lactic dehydrogenase, LDH (*ca.* 5 mg. protein/ml.):
Dilute the enzyme suspension containing *ca.* 10 mg. protein/ml. in 2.1 M ammonium sulphate solution with distilled water.

Stablity of the solutions

Store all solutions, stoppered, in a refrigerator at 0—4°C. The DPN solution is stable for several weeks and need not be neutralized because of the high buffering capacity of the hydrazine-glycine buffer. The hydrazine-glycine buffer is only stable for a week; it is better to prepare a stock solution containing hydrazine sulphate, glycine and disodium EDTA. This is stable virtually indefinitely and small portions can be adjusted to pH 9.5 as required.

Procedure

Experimental material

Obtain blood without constriction of the vein and immediately deproteinize. For studies on plasma, centrifuge off the erythrocytes in the cold as soon as possible after obtaining the blood[3]. Freeze tissue samples within a fraction of a second[3] and do not allow to thaw until ready to deproteinize.

Deproteinization

Preliminary remarks: Add perchloric acid to deproteinize the sample. There are two possible methods of extraction: single extraction and calculation of the volume of the extract on the basis of an assumed, mean water content of the sample (see p. 254), or repeated and therefore quantitative extraction of the tissue. The first method is suitable when only lactate is to be determined. The second method is preferable when other metabolites, which are difficult to extract (*e.g.* organic phosphoric acid derivatives), are to be estimated in the same extract. It is convenient always to use the same ratio of volume of extract to tissue weight of 8 : 1. Use the following amounts of perchloric acid for the single extraction method:

7.2 ml. perchloric acid solution to 1 g. of blood
7.15 ml. perchloric acid solution to 1 ml. of blood
7.25 ml. perchloric acid solution to 1 g. of tissue

With repeated extraction it is usually sufficient to extract twice and to dilute the extract to the volume to weight ratio of 8 : 1. In this case the error due to retention of the compound in the precipitate is not more than 3—4%.

[3] *H. J. Hohorst, F. H. Kreutz* and *Th. Bücher*, Biochem. Z. *332*, 18 [1959].

Method: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 1 g. of sample (allow blood to flow directly from the cannula; powder frozen tissue[3]), mix quickly and re-weigh.

Single extraction: From the increase in weight (= weight of tissue) calculate the total volume of perchloric acid required (see above) and then add the requisite quantity of perchloric acid solution (III) to the 5 ml. already present. Mix the suspension thoroughly. Grind lumps of tissue on the walls of the tube with a glass rod and then centrifuge at a minimum of 3000 g for 5 min. Transfer the supernatant to a cooled 10 ml. flask for neutralization.

Repeated extraction: Thoroughly mix the suspension of the sample in the 5 ml. perchloric acid (in special cases use a homogenizer) and immediately centrifuge at 3000 g. Decant the supernatant, stir the precipitate with 1 ml. perchloric acid solution (III) + 1 ml. distilled water and re-centrifuge. Combine the supernatants, measure the volume and make up to 8 ml./g. with distilled water.

Neutralization: Pipette 0.02 ml. indicator solution (II) into 8 ml. extract and, while stirring vigorously with a magnetic stirrer and cooling in ice, add about 0.1 ml. carbonate solution (I) from a 1 ml. graduated pipette. Wait until the CO_2 evolution has nearly ceased and then add more carbonate solution until the mixture is salmon-pink (pH *ca.* 3.5). A total of about 0.16 ml. carbonate solution is required. Allow to stand for 10 min. in an ice bath, decant or pipette the supernatant from the precipitated perchlorate and use a measured portion for the determination.

Spectrophotometric measurements

Preliminary remarks: The ratio of the total volume to the sample volume must not exceed 2:1 so that the hydrazine-glycine buffer is not diluted too much. It is convenient always to use the same dilution ratio so that, in calculating the results, the optical density difference need only be multiplied by a constant factor. A control cuvette, which contains the same solutions as the experimental cuvette except the sample, is necessary. The reason is that DPN and hydrazine form a complex (see footnote on p. 385). This complex reacts comparatively slowly with LDH *) to form a compound which absorbs in the near ultraviolet more strongly than the DPN-hydrazine complex.

Method:

Wavelength: 340 or 334 mμ; light path: 1 cm.; final volume 1.01 ml. Measure against the control cuvette.

Experimental cuvette	*Control cuvette*
0.45 ml. hydrazine-glycine buffer (IV)	0.45 ml. hydrazine-glycine buffer (IV)
0.05 ml. DPN solution (V)	0.05 ml. DPN solution (V)
0.10 ml. deproteinized extract	0.50 ml. distilled water
0.40 ml. distilled water	

Wavelength: 366 mμ; light path: 1 cm.; final volume: 1.01 ml. Measure against the control cuvette.

*) Not with glutamic dehydrogenase, malic dehydrogenase or glycerol-1-phosphate dehydrogenase.

Experimental cuvette	*Control cuvette*
0.45 ml. hydrazine-glycine buffer (IV)	0.45 ml. hydrazine-glycine buffer (IV)
0.05 ml. DPN solution (V)	0.05 ml. DPN solution (V)
0.30 ml. water	0.50 ml. distilled water
0.20 ml. deproteinized extract	

Mix thoroughly, allow the cuvette contents to warm to room temperature and read the optical density E_1 twice with an interval of 3 min. Mix into the experimental cuvette

0.01 ml. LDH suspension (VI).

On completion of the reaction (10–20 min. after addition of the enzyme, depending on the lactate concentration) read the optical density E_2 twice with an interval of 3 min. The changes in the initial optical density E_1 and in the final optical density E_2 are usually small in comparison with the optical density difference $\Delta E = E_2 - E_1$ and can be neglected. The ΔE at 340 mμ should not be more than 1.0 (corresponding $\Delta E_{366} \approx 0.53$). If E_2 is still increasing 20 min. after addition of the enzyme, although the initial optical density E_1 was constant for at least 3 min., then providing no other interfering substances are present (see below), the activity of the lactic dehydrogenase is too low.

To check that the assay is functioning correctly, mix 0.01 ml. 0.002 M L-lactate solution into the experimental cuvette on completion of the reaction. The increase in optical density should be complete in 10–20 min. Read the optical density E_3 twice within 3 min. and calculate the difference $\Delta E' = E_3 - E_2$. Under the stated conditions $\Delta E'$ should be 0.123 at 340 mμ and 0.065 at 366 mμ.

Calculations

L-(+)-Lactate reacts quantitatively, so that the amount in the sample can be calculated from the optical density difference:

$$\frac{\Delta E \times \text{dil.}}{\varepsilon \times d} = \mu\text{moles L-(+)-lactate/g. tissue}$$

where

ΔE = optical density difference ($E_2 - E_1$)
dil. = total dilution of the sample
ε = extinction coefficient [cm.²/μmole]
d = light path [cm.]

With constant dilution ratios the equation simplifies to

$$\Delta E \times F = \mu\text{moles L-(+)-lactate/g. tissue} \quad \left(F = \frac{\text{dil.}}{\varepsilon \times d}\right)$$

When:

the ratio of volume of extract to weight of tissue is 8 : 1,
volume of neutralized extract to weight of tissue is 8.2 : 1,
dilution of the extract in the assay is 10.1 : 1 (at 334 and 340 mμ); 5.05 : 1 (at 366 mμ) and the total dilution is 82.8 : 1 (at 334 and 340 mμ); 41.4 : 1 (at 366 mμ)

Then the values for F are: 334 mμ: 13.6
340 mμ: 13.2
366 mμ: 12.5

At 366 mμ the extinction coefficient is slightly dependent on the temperature: $\varepsilon\,{}^{366\,m\mu}_{25°C} = 3.3$ cm.²/μmole; $\varepsilon\,{}^{366\,m\mu}_{1°C} = 3.6$ cm.²/μmole. The values given here for F are for 25° C.

Example

1.348 g. of blood from a healthy person were added to 5 ml. perchloric acid. To obtain the ratio of 1 g. blood to 7.2 ml. perchloric acid the mixture was made up to $1.348 \times 7.2 = 9.72$ ml. with perchloric acid solution and neutralized. Measurements at 340 mμ against a control cuvette; light path: 1 cm.

Before addition of LDH	0 min.	$E_1 = 0.083$
	3 min.	$E_1 = 0.084$
After addition of LDH	12 min.	$E_2 = 0.187$
	15 min.	$E_2 = 0.188$

$\Delta E = E_2 - E_1 = 0.187 - 0.083 = 0.104$

$0.104 \times 13.1 = 1.36$ μmoles L-(+)-lactate/g. blood.

Other Determinations

Other metabolites can be determined in the same assay mixture by the addition of specific enzymes[3], *e.g.* L-(−)-glycerol-1-phosphate (α-glycerophosphate) and L-(−)-malate.

Sources of Error

1. A constant end-point is not reached within 30 min. if the activity of the lactic dehydrogenase is too low. Check the activity of the enzyme and, if necessary, use larger amounts of enzyme or a fresh preparation.

2. The initial optical density is not constant if: a) the cuvette contents were not brought to room temperature before beginning the measurements, b) the hydrazine-glycine buffer is more than 8 days old, c) the DPN preparation is impure or d) a change occurs in the absorption due to the tissue extract. In the last case, measure against a control cuvette, which contains the same solutions as the experimental cuvette, but to which no enzyme is added.

3. The addition of the enzyme causes a sharp change in optical density. If the optical density increases then the enzyme usually has too high an absorption and therefore a new enzyme preparation should be used. If the optical density decreases then the hydrazine-glycine buffer is too alkaline. At pH ≥ 9.6 the initial optical density of the assay mixture is higher. Addition of the enzyme includes addition of ammonium sulphate, which lowers the pH and so causes a sharp decrease in optical density.

4. The optical density reaches a maximum and then falls (especially at a higher temperature, *e.g.* 37°C), because of the autoxidation of DPNH[4]. The remedy is to evacuate the experimental cuvette.

Specificity

Only L-(+)-lactate (sarcolactic acid) reacts. D-Lactate does not react. The racemate only reacts to 50% of the amount present. Apart from L-(+)-lactate, α-hydroxybutyrate and β-chlorolactate react to a slight extent. In equimolar amounts these compounds, which do not occur naturally, cause an elevation of the analytical results by 8.4 and 6.3% respectively.

Other Methods for the Determination of L-(+)-Lactate

Other methods for the enzymatic determination of L-lactate have been described by *Horn* and *Bruns*[5], *Hess*[6], *Pfleiderer* and *Dose*[7], and also *Wieland*[8]. For the determination with LDH and the acetylpyridine analogue of DPN, see p. 275.

4) *H. J. Hohorst*, Biochem. Z. *328*, 509 [1957].
5) *H. D. Horn* and *F. H. Bruns*, Biochim. biophysica Acta *21*, 378 [1956].
6) *B. Hess*, Biochem. Z. *328*, 110 [1956].
7) *G. Pfleiderer* and *K. Dose*, Biochem. Z. *326*, 436 [1955].
8) *G. Wieland*, Biochem. Z. *329*, 568 [1958].

Determination with Lactic Dehydrogenase from Yeast

Otto Wieland

The determination of lactate with the DPN-linked lactic dehydrogenase (LDH) from muscle has some disadvantages, for example, the length of time required for completion because of the unfavourable position of the equilibrium. The determination of lactate with the yeast LDH which is not DPN-linked[1,2] has proved successful as a routine method. By this method lactic acid can be determined in the presence of a large excess of pyruvate[3].

Principle

Yeast LDH, a flavocytochrome[4-6], transfers hydrogen from lactate to potassium ferricyanide:

(1) $CH_3-CHOH-COOH + 2\,[Fe(CN)_6]^{3-} \longrightarrow CH_3-CO-COOH + 2\,[Fe(CN)_6]^{4-} + 2\,H^+$

The decrease in colour on reduction of the ferricyanide ion can be followed at 405 mμ. The absorption maximum of ferricyanide in the visible spectrum is at 420 mμ. As ferrocyanide does not react with oxygen the measurements can be made in open cuvettes.

Reagents

1. Potassium ferricyanide, $K_3[Fe(CN)_6]$, A. R.
2. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10\,H_2O$, A. R.
3. Hydrochloric acid, A. R., 2 N
4. Lactic acid (racemate)
   1 M aqueous solution or Na or Li salt
5. Lactic dehydrogenase, LDH
   from yeast (purified at least 20 to 25-fold). For the preparation of the enzyme according to[4], see the Appendix, p. 274.
6. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)
7. Potassium hydroxide, A. R., 1 N

Preparation of Solutions

I. Potassium ferricyanide (0.01 M):
Dissolve 3.2925 g. $K_3[Fe(CN)_6]$ in distilled water and make up to 1000 ml.

II. Pyrophosphate buffer (0.07 M; pH 8.0):
Dissolve 31.2 g. $Na_4P_2O_7 \cdot 10\,H_2O$ in about 900 ml. distilled water, adjust to pH 8.0 with 2 N HCl and dilute to 1000 ml. with distilled water.

III. Lactate standard solution (450 μg. L-(+)-lactate/ml.):
Adjust 1 ml. 1 M DL-lactic acid to pH 7.5 with 0.1 N NaOH and dilute to 100 ml. with distilled water, or dissolve 112.08 mg. Na-DL-lactate or 96.02 mg. Li-DL-lactate in distilled water and make up to 100 ml.

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1) *J. Lehmann*, Scand Arch. Physiol. *80*, 237 [1938].
2) *O. Wieland*, Biochem. Z. *329*, 568 [1958].
3) *K. Wallenfels* and *D. Hofmann*, Tetrahedron Letters *1959*, No. 15, p. 10.
4) *M. Dixon* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 444.
5) *C. A. Appleby* and *R. K. Morton*, Nature [London] *173*, 769 [1954].
6) *A. P. Nygaard*, Biochim. biophysica Acta *30*, 450 [1958]; *33*, 517 [1958]; *35*, 212 [1959].

IV. Lactic dehydrogenase, LDH (30—50 mg. protein/ml.):
Dilute the aqueous solution obtained from yeast with distilled water.

V. Perchloric acid (3% w/v):
Dilute 2.6 ml. $HClO_4$ (70% w/w) to 100 ml. with distilled water.

Stability of the solutions

Store the lactate standard solution in a refrigerator. The enzyme solution keeps for several months at −20° C. The other solutions can be stored at room temperature.

Procedure

Experimental material

With the method described here lactate can be determined in blood (serum, plasma), cerebrospinal fluid, organ extracts, *etc.* Collect blood without excessive constriction of the veins. To obtain plasma add 4 mg. NaF/ml. blood. Remove organs as quickly as possible and chill so as to avoid postmortem changes in the constituents. Preferably use the "quick-freeze" method (refer to p. 47).

Deproteinization

With the exception of solutions extremely low in protein, such as urine, serous discharges or cerebrospinal fluid, the samples must be deproteinized. To 1 ml. sample add 2 ml. 3% (w/v) perchloric acid (solution V), centrifuge and neutralize 2 ml. of the supernatant with 1 N KOH. After allowing to stand for a short period in an ice bath, centrifuge off the precipitate and dilute the supernatant to 3 ml. with distilled water. To extract liver tissue, homogenize 1 part by weight with 3 parts by volume 3% (w/v) perchloric acid (solution V) in a glass homogenizer with cooling, then centrifuge and neutralize the supernatant with 1 N KOH.

The method has also been used for the micro-determination of lactic acid in blood taken from the finger: collect 0.1 ml. blood in a blood sugar pipette and immediately pipette into 1 ml. 3% (w/v) perchloric acid (solution V).

Spectrophotometric measurements

Wavelength: 405 mμ; light path: 1 cm.; final volume: 2 ml. Measure against the control cuvette.

Pipette into the cuvettes:

Experimental cuvette	*Control cuvette*
1.00 ml. buffer (solution II)	1.00 ml. buffer (solution II)
0.15 ml. ferricyanide solution (I) *)	0.15 ml. ferricyanide solution (I)
0.50 ml. deproteinized sample	0.50 ml. deproteinized sample
distilled water to 2.00 ml.	distilled water to 2.00 ml.

Read the optical density E_1. Mix into the experimental cuvette
0.01—0.02 ml. LDH solution (IV).

On completion of the reaction read the optical density E_2. To determine the small absorption due to the LDH solution, once again add the same volume of LDH solution (IV). Correct E_2 for any increase in optical density.

*) In the Legend to Fig. 1 of the original communication [2] 0.015 ml. was given in error.

For a series of determinations prepare the assay mixtures in test tubes and read E_2 after 10 to 15 min. incubation at 30° C.

To check the method set up standards containing 0.1—0.5 ml. lactate standard solution (III) instead of the deproteinized sample. The amounts of lactate added (45—225 μg.) should be recovered with an error of ±1%.

Modification for micro-determinations

Since in micro-analyses insufficient deproteinized sample is available for a control cuvette, the measurements are made against air. The optical density must therefore be followed until a constant rate is given or until the reaction stops (refer to p. 269)[7].

Narrow cuvettes are used (light path: 2 cm.). The assay mixture contains:

0.94 ml. buffer (solution II)
0.05 ml. ferricyanide solution (I)
1.00 ml. deproteinized sample
distilled water to 2.00 ml.

Start the reaction by the addition of

0.01—0.02 ml. LDH solution (IV).

Calculations

According to equation (1) 2 moles ferricyanide are equivalent to 1 mole L-(+)-lactate.

Therefore

$$\frac{\Delta E \times 2}{0.96 \times 2} = \frac{\Delta E}{0.96} = \text{μmoles L-(+)-lactate in the reaction mixture}$$

where

$\Delta E = E_1 - E_2$ (E_2 corrected for the LDH absorption)

0.96 = extinction coefficient for $K_3[Fe(CN)_6]$ [cm.²/μmole]

2 (numerator) = volume of the assay mixture [ml.]

With cuvettes having a light path of 2 cm. the formula is:

$$\frac{\Delta E}{0.96 \times 2} = \text{μmoles L-(+)-lactate in the reaction mixture.}$$

To calculate the lactate content of the starting material it is necessary to multiply by the dilution factors for the deproteinization and the reaction mixture.

Example

$\Delta E = 0.317$; therefore it follows:

$$\frac{0.317}{0.96} = 0.33 \text{ μmoles lactate/reaction mixture.}$$

0.5 ml. of a deproteinized supernatant was taken for the assay. The volume of the supernatant (3.0 ml.) was $^2/_3$ of that of the blood taken.

Therefore:

$$0.33 \times \frac{3}{0.5} \times \frac{3}{2} = 2.97 \text{ μmoles lactate/ml. blood.}$$

To obtain the results in the usual clinical units of mg.% they must be multiplied by the factor 9, which includes the molecular weight of lactic acid (90); for example:

$$2.97 \times 9 = 26.73 \text{ mg.\% lactic acid in blood.}$$

[7] The Eppendorf photometer with an automatic recording attachment (Netheler & Hinz, Hamburg, Germany) was used in this work.

Sources of Error

Biological samples may contain compounds (ascorbic acid, glutathione, cysteine, DPNH) which reduce ferricyanide. These compounds do not interfere in the determination when the measurements are made against a control cuvette containing the sample. With LDH preparations which are not sufficiently pure a reduction of ferricyanide is observed, which cannot be attributed to the presence of lactate. In this case the blank reaction for the enzyme preparation is determined separately and the experimental results are corrected accordingly. However, it is better to use a more highly purified enzyme preparation.

Specificity

The yeast enzyme, like the animal LDH, does not only react with lactic acid. α-Hydroxybutyric acid reacts at about half the rate of lactate, while glycollic acid, glyceric acid and α-glycerophosphate react at considerably slower rates[1,2]. However, in most cases these substrates occur in such low concentrations in comparison to lactic acid, that under the conditions described here practically no interference occurs. Only the L-(+)-optical isomer of lactic acid reacts. Even in high concentration D-(−)-lactate is not attacked by the yeast LDH used in these studies[2]. Recently yeast LDH has been separated chromatographically into several components, one of which also reacts with D-(−)-lactate[6].

Appendix

Isolation of Yeast LDH

Lactic dehydrogenase (LDH) was purified from baker's yeast according to the description of *Dixon*[4] (with certain changes). Starting material: "Oberkotzauer" yeast or "Giegold" yeast. The LDH content of the yeasts differs considerably and should be examined before commencing the purification. The heating step described in the original publication[4] could not be reproduced and was therefore omitted.

1. Toluene autolysis

Warm 650 g. fresh yeast to *ca.* 37°C in a glass beaker and mix with 40 ml. toluene. After incubating for about 1 hour at 37°C with occasional stirring, the yeast liquefies. Add 30 ml. water, incubate for 45 min. at 37°C, add a further 650 ml. water, stir and adjust the pH to between 7.5 and 8 with 2 N KOH. Allow to stand for 48 hours at room temperature and for 48 hours at 2 to 4°C with occasional stirring. Centrifuge at 8000 to 10000 g. The yellow-brown crude extract (750 ml.; specific activity: 187 units/mg.) can be stored overnight at 2°C without loss of activity.

2. Adsorption of inactive protein on aluminium hydroxide gel

Inactive protein is removed by adsorption on 2% alumina-C_γ-gel. Determine the required amounts of gel in preliminary tests. The isolation described here required 240 ml. gel for 750 ml. crude extract (= 14.4 g. protein). After centrifuging off the gel the supernatant still contained 95% of the initial activity.

3. Adsorption on phosphate gel

To the supernatant from step 2 (810 ml.) add 250 ml. phosphate gel prepared according to *Kunitz*[8] (determine the amounts of gel in preliminary tests) and then centrifuge. Discard the supernatant (contains only *ca.* 5% of the activity). Wash the gel sediment with four 100 ml. portions of M/15 potassium phosphate buffer (pH 7).

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[8] *M. Kunitz*, J. gen. Physiol. *35*, 340 [1952].

4. Elution

Elute the sediment twice with 100 ml. and once with 50 ml. M/15 potassium phosphate buffer (pH 7 containing 10% ammonium sulphate) and combine the eluates. They contain 80% of the LDH activity present in the supernatant from step 2.

5. Ammonium sulphate fractionation

Slowly add, with stirring, 55 g. solid $(NH_4)_2SO_4$ to the eluate from step 4 (255 ml.). After allowing to stand for 30 min. in an ice bath, centrifuge off the precipitate and discard. To the clear supernatant add 60 g. ammonium sulphate, centrifuge off the precipitate as described above and dissolve in 0.001 M ethylene-diamine-tetra-acetate. This LDH fraction is 20 to 25-fold purified as compared to the crude extract (specific activity: 4300 units/mg.). A further purification can be obtained by repetition of the gel steps (2, 3 and 4). Repetition of the fractionation with ammonium sulphate at pH 4.8 also leads to further purification.

Determination of enzyme activity

The assay mixture contains:

1.00 ml. 0.07 M sodium pyrophosphate buffer (solution II)
0.15 ml. 0.01 M $K_3[Fe(CN)_6]$ solution (I)
0.05 ml. 0.10 M Na-DL-lactate solution
distilled water + sample to 2.00 ml.

Light path: 1 cm.; wavelength: 405 mμ; temperature: 25° C (constant). Observe the optical density changes without lactate and after the preliminary reaction has stopped start the actual assay by mixing in the lactate solution. Read the optical density at 30 sec. intervals. An LDH unit is the amount of enzyme which causes an optical density change of $\Delta E = 0.001$ in the first minute.

Determination with Lactic Dehydrogenase and the 3-Acetylpyridine Analogue of DPN (AP-DPN)

Helmut Holzer and Hans-Dieter Söling

Replacement of diphosphopyridine nucleotide (DPN) by the 3-acetylpyridine analogue alters the position of the equilibrium of DPN-dependent enzyme reactions[1, 2]. The $AP\text{-}DPN^+$/AP-DPNH system, which has a more positive redox potential than the DPN^+/DPNH system, displaces the equilibrium in favour of oxidation of the substrate. Therefore 3-acetylpyridine-DPN can be used for spectrophotometric determinations (based on *Warburg*'s principles[3]), without the need for trapping the oxidation product.

Principle

Lactic dehydrogenase (LDH) from rabbit muscle catalyses the reaction:

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

At pH 7 the equilibrium lies to the left, but at pH 10 the equilibrium constant (omitting the H^+ions) is about 1 (see Appendix). Alkaline pH therefore favours the oxidation of lactic acid. Since the affinity of LDH for L-lactic acid in the assay with AP-DPN is relatively low ($K_M = 2.9 \times 10^{-3}$ M), high concentrations of LDH and AP-DPN must be used to obtain a quantitative oxidation of L-lactic acid.

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[1] *N. O. Kaplan* and *M. M. Ciotti*, J. biol. Chemistry *221*, 823 [1956].
[2] *N. O. Kaplan, M. M. Ciotti* and *F. E. Stolzenbach*, J. biol. Chemistry *221*, 833 [1956].
[3] *O. Warburg:* Wasserstoffübertragende Fermente. Verlag Werner Saenger, Berlin 1948.

Reagents

1. Glycine, A. R.
2. Sodium hydroxide, A. R., 2 N
   prepare freshly each day with carbonate-free, doubly distilled water.
3. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (*e.g.* Titriplex III from E. Merck, Darmstadt, Germany).
4. 3-Acetylpyridine analogue of diphosphopyridine nucleotide, AP-DPN *)
5. Lactic dehydrogenase, LDH
   crystalline, from rabbit muscle, suspended in 2.2 M ammonium sulphate solution. Commercial preparation, see p. 986.

Preparation of Solutions

I. Glycine buffer (1 M; pH 9.5):
Dissolve 7.5 g. glycine and 186 mg. EDTA-$Na_2H_2 \cdot 2H_2O$ in 12 ml. 2 N NaOH and 30 ml. doubly distilled water. Adjust to pH 9.5 (glass electrode) with *ca.* 2 ml. 2 N NaOH and dilute to 100 ml. with doubly distilled water.

II. 3-Acetylpyridine analogue of diphosphopyridine nucleotide (*ca.* 8×10^{-3} M AP-DPN):
Dissolve 5 mg. AP-DPN in 1.0 ml. doubly distilled water

III. Lactic dehydrogenase, LDH (5 mg. protein/ml.):
Dilute crystalline suspension with 2.2 M ammonium sulphate solution.

Procedure

For preparation and extraction of the samples (blood, tissue, *etc.*), see p. 254.

Spectrophotometric measurements

Wavelength: 366 mμ; glass cuvettes. The absorption maximum of AP-DPN is at 363 mμ ([1,4], in agreement with our own measurements).

To conserve the expensive AP-DPN use semi-microcuvettes with a total volume of 0.4 ml. and a light path of 0.5 cm. By variation of the light path and the final volume the assay can be made more sensitive. Measure against distilled water. Bring the buffer and solution of sample to room temperature.

Pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
0.31 ml. buffer (solution I)	0.31 ml. buffer (solution I)
0.04 ml. AP-DPN solution (II)	0.04 ml. AP-DPN solution (II)
sample + water to 0.39 ml.	water to 0.39 ml.

Record the optical density of both cuvettes. If the optical density change in both cuvettes is not more than 0.001 to 0.002 per 30 sec., mix

0.01 ml. LDH suspension (III) (*ca.* 50 μg protein)

into both cuvettes. After 15—25 min. the increase in optical density with time is very small and is the same in the experimental and control cuvette. Controls, containing all the components of the assay except the enzyme, usually show no significant optical density change

*) Obtainable from The Pabst Laboratories, Milwaukee, Wisconsin, USA.

[4] *J. M. Siegel, G. A. Montgomery* and *R. M. Bock*, Arch. Biochem. Biophysics *82*, 288 [1959].

with time. The optical density difference between the experimental and control cuvette on completion of the reaction minus the optical density difference between the experimental and control cuvette before the start of the reaction with LDH gives the ΔE required for the calculations.

Calculations

$$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles L-lactate in the cuvette}$$

ΔE is the increase in optical density on addition of LDH, corrected as described above. The extinction coefficient of AP-DPNH is 9.1 cm.^{2}/μmole[4] at 366 mμ, d is the light path of the cuvette in cm. and V is the final volume of the assay mixture in ml.

Appendix [6]

Initial velocities of lactate oxidation with AP-DPN or DPN

With 5×10^{-5} M AP-DPN or DPN, in 0.68 M glycine buffer pH 9.5 and with 50 mg. protein/l., the ratio of the initial velocities of lactate oxidation V_{DPN}: $V_{AP\text{-}DPN}$ is 7.2 : 1.

Michaelis constants

The rates of reaction with 4.6×10^{-5} to 1.16×10^{-3} M lactate and 5×10^{-6} to 1×10^{-4} M AP-DPN were measured in 0.9 M glycine buffer (pH 9.5) at 24°C. The constants were calculated according to *Lineweaver* and *Burk*[5].

With 7.5×10^{-3} M lactate the K_M for AP-DPN is 4.7×10^{-5} M; with 3.3×10^{-4} M AP-DPN the K_M for lactate is 2.9×10^{-3} M.

The equilibrium constant $K = \frac{[\text{AP-DPNH}] \times [\text{pyruvate}] \times [H^+]}{[\text{AP-DPN}^+] \times [\text{lactate}]}$ is 5.65×10^{-10} moles/l. at 25°C (mean of four determinations). Lactate was determined by the assay method described above. AP-DPN was estimated by quantitative conversion to AP-DPNH with malic dehydrogenase and saturation with malate at pH 9.5 (refer to p. 332). The H^+ concentration was measured in the cuvette with a glass electrode after the equilibrium had been attained. The equilibrium concentrations of AP-DPN and pyruvate were obtained from the spectrophotometrically measured AP-DPNH concentration at equilibrium.

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[5] *H. Lineweaver* and *D. Burk*, J. Amer. chem. Soc. *56*, 658 [1934].
[6] *H. Holzer* and *H. D. Söling*, Biochem. Z. *336*, 201 [1962].