L-(—)-Malate

Determination with Malic Dehydrogenase and DPN

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Principle

Malic dehydrogenase (MDH) catalyses the oxidation of L-(—)-malate by diphosphopyridine nucleotide (DPN).

(1) L-(—)-Malate + DPN^+ ⇌ oxaloacetate + DPNH + H^+

The equilibrium of the reaction, which lies far to the left, has a constant K_C of 5.0×10^{-13} moles/l[1] at 25°C. To obtain quantitative oxidation of L-(—)-malate the reaction products must be removed. Protons are bound by the use of an alkaline reaction medium and oxaloacetate is trapped as the hydrazone. Therefore the basic equation for the spectrophotometric assay of L-(—)-malate is:

(2) L-(—)-Malate + DPN^+ + hydrazine $\xrightarrow[\text{pH 9.5}]{\text{MDH}}$ oxaloacetate hydrazone + DPNH + H_3O^+

Relatively high concentrations of DPN and MDH are necessary to obtain a quantitative and sufficiently fast reaction (see under "Sources of Error"). The course of the reaction is followed spectrophotometrically (increase in optical density due to 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. Malic dehydrogenase, MDH
   from pig heart, suspension in 2.8 M ammonium sulphate solution. Commercial preparation, see p. 988.

   Purity of the enzyme preparation

   The MDH preparation should have a specific activity of at least 2000 units/mg. (according to *Bücher**) or 36 units/mg. (according to *Racker**). Contamination by lactic dehydrogenase or glycerol-1-phosphate dehydrogenase should not exceed 0.05% (relative to the MDH activity).

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.

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*) Definition of units see p. 32 and 33.

[1] *H. J. Hohorst*, Ph. D.-Thesis, Universität Marburg, 1960.

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 *ca.* 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. Malic dehydrogenase, MDH (*ca.* 5 mg. protein/ml.):
Dilute the enzyme suspension containing *ca.* 10 mg. protein/ml. in 2.8 M ammonium sulphate solution with distilled water.

Stability 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 alkaline 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 practically indefinitely and small portions can be adjusted to pH 9.5 as required.

Procedure

Experimental material

Freeze tissue samples within a fraction of a second[2] 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 malate is to be determined and when the tissue is easily disintegrated, *e.g.* liver. The second method is preferable when other metabolites are to be determined in the same extract. With a single extraction the ratio of the volume of extract to the tissue weight should be 4 : 1. If the tissue is assumed to have a water content of 75%, then

6.5 ml. perchloric acid solution (III)

are added to 2 g. tissue. With repeated extraction the ratio of the volume of extract to the tissue weight should be 8 : 1. Usually it is sufficient to extract twice and to dilute the extract to the corresponding 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%.

Method:

Single extraction: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 2 g. of tissue (frozen powder)[2], mix quickly and re-weigh. 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 the perchloric acid solution (III)

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

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 centrifuge for 5 min. at 3000 g. Transfer the supernatant to a cooled 10 ml. flask for neutralization.

Repeated extraction: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 1 g. of tissue (frozen powder), mix quickly and re-weigh. If necessary homogenize the material. Centrifuge for about 5 min. 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 add distilled water to give 8 ml./g. sample.

Neutralization: Pipette 0.02 ml. indicator solution (II) into each 8 ml. of tissue extract and, while stirring vigorously with a magnetic stirrer and cooling in ice, add 0.1 ml. carbonate solution (I) from a 1 ml. graduated pipette. Wait until the CO_2 evolution has practically ceased and then add more carbonate solution until the mixture is salmon-pink (*ca.* pH 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 off the supernatant from the precipitated perchlorate and use a portion for the determination.

Spectrophotometric measurements

Preliminary remarks: The ratio of the total volume to the volume of the sample should not exceed 2:1 so that the hydrazine-glycine buffer is not diluted too much. It is convenient to always choose the same dilution ratio so that, in calculating the results, the optical density differences need only be multiplied by a constant factor. A control or blank cuvette can usually be omitted, therefore the measurements are made against air or water (see under "Sources of Error").

Method:

Wavelength: 340 or 334 mμ; light path: 1 cm.; final volume 1.01 ml.

Experimental cuvette	*Control cuvette*
0.45 ml. hydrazine-glycine buffer (IV)	water or in special cases (see under "Sources of Error") as for experimental cuvette.
0.05 ml. DPN solution (V)	
0.50 ml. deproteinized extract	

Wavelength: 366 mμ; light path: 2 cm.; final volume: 2.02 ml.

Experimental cuvette	*Control cuvette*
0.9 ml. hydrazine-glycine buffer (IV)	water or in special cases as for experimental cuvette
0.1 ml. DPN solution (V)	
1.0 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.*) MDH solution (VI).

On completion of the reaction (10—20 min. after enzyme addition, depending on the malate 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 normally small in comparison with the difference in optical density $\Delta E = E_2 - E_1$ and can be neglected.

*) or 0.02 ml. for an assay volume of 2.02 ml.

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 the addition of the enzyme, while the initial optical density E_1 was constant for at least 3 min., then as long as no other interfering substances are present (see below), the activity of the malic dehydrogenase is probably too low.

To check that the assay is working correctly, mix 0.01 ml. 0.002 M L-(−)-malate 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-(−)-Malate reacts quantitatively, so that the amount in the sample can be calculated from the optical density difference

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

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

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

If the ratio of volume of extract to weight of tissue is 4 : 1 then:
volume of the neutralized extract to weight of tissue is 4.1 : 1,
dilution of extract in the assay is 2.02 : 1 and
total dilution is 8.28 : 1.

Giving values for F at 334 mμ: 1.36
340 mμ: 1.32
366 mμ: 1.25

If the ratio of volume of extract to weight of tissue is 8 : 1 then:
volume of the neutralized extract to weight of tissue is 8.2 : 1,
dilution of the extract in the assay is 2.02 : 1 and
total dilution is 16.5 : 1.

Giving values for F at 334 mμ: 2.72
340 mμ: 2.64
366 mμ: 2.51

At 366 mμ the extinction coefficient is dependent on the temperature (see p. 27). The values given here for F_{366} are for 25° C.

Example

1.276 g. tissue (rat liver) were added to 5 ml. perchloric acid. After extracting twice, the volume of extract was 7.6 ml. This was made up to 10.2 ml. with 2.6 ml. perchloric acid (volume of extract: weight of tissue = 8 : 1) and neutralized.

Measurements at 334 mμ: light path = 1 cm.; against control cuvette containing water.

Before addition of MDH 0 min. $E_1 = 0.148$
3 min. $E_1 = 0.150$
After addition of MDH 10 min. $E_2 = 0.241$
13 min. $E_2 = 0.243$

$\Delta E = E_2 - E_1 = 0.241 - 0.148 = 0.093$.
$0.093 \times 2.72 = 0.253$ μmoles L-malate/g tissue.

Further Determinations

Other metabolites, *e.g.* L-(+)-lactate and L-(−)-glycerol-1-phosphate, can be determined in the same assay mixture by the addition of specific enzymes [2].

Sources of Error

1. If a constant end-point is not reached within 30 min. the activity of the malic 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 are 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 optical density change. If the optical density increases then usually the enzyme has too high an absorption, so therefore use a new enzyme preparation. If the optical density decreases then the hydrazine-glycine buffer may be 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 value and so causes a sharp decrease in optical density.

4. The optical density reaches a maximum and then falls (especially at higher temperatures, *e.g.* 37° C) because of autoxidation [3] of the DPNH. The remedy is to evacuate the experimental cuvette.

Specificity

The assay is specific for L-(−)-malate. The dextrorotatory isomer does not react. The racemate only reacts to 50% of the amount present. L-Lactate, D-lactate, aspartate and fumarate do not react.

Determination with Malic Dehydrogenase and the 3-Acetylpyridine Analogue of DPN

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The spectrophotometric determination [1] of L-malate with malic dehydrogenase from pig heart muscle and the 3-acetylpyridine analogue of DPN (AP-DPN), without the use of a trapping agent for oxaloacetate, is made possible by the favourable redox potential of $AP\text{-}DPN^+/AP\text{-}DPNH$ in contrast to that of $DPN^+/DPNH$ (see p. 334, also literature concerning AP-DPN).

Principle

Malic dehydrogenase (MDH) catalyses the reaction:

$$\text{L-Malate} + \text{AP-DPN}^+ \rightleftharpoons \text{oxaloacetate} + \text{AP-DPNH} + \text{H}^+$$

[3] *H. J. Hohorst*, Biochem. Z. *328*, 509 [1957].

[1] *O. Warburg:* Wasserstoffübertragende Fermente. Verlag Dr. Werner Saenger GmbH, Berlin 1948.

The equilibrium at pH 7 is to the left, while at pH 9 the apparent equilibrium constant (without taking into account H^+ ions) is about 1 (see Appendix, p. 334). Therefore an alkaline pH favours the oxidation of malate. The affinity of MDH for L-malate in the test with AP-DPN is high ($K_M = 4.1 \times 10^{-4}$ M).

Reagents

1. Glycine, A. R.
2. Sodium hydroxide, A. R., 2 N
3. Ethylene-diamine-tetra-acetic acid, A. R., EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2\ H_2O$.
4. 3-Acetylpyridine analogue of diphosphopyridine nucleotide, AP-DPN *)
5. Malic dehydrogenase, MDH
   from pig heart muscle, suspended in 2.8 M ammonium sulphate solution. Commercial preparation, see p. 988.

Preparation of Solutions

I. Glycine buffer (1 M; pH 9.5):
Dissolve 7.5 g. glycine + 186.13 mg. EDTA-$Na_2H_2 \cdot 2H_2O$ in 30 ml. doubly distilled water and 12 ml. 2 N NaOH. Adjust pH to 9.5 with *ca.* 2 ml. 2 N NaOH (glass electrode) and dilute to 100 ml.

II. 3-Acetylpyridine analogue of diphosphopyridine nucleotide (*ca.* 2×10^{-3} M AP-DPN):
Dissolve 2 mg. AP-DPN in 1.0 ml. doubly distilled water.

III. Malic dehydrogenase, MDH (0.5 mg. protein/ml.):
Use suspension of *ca.* 5 mg. protein in 10 ml. 2.8 M ammonium sulphate solution; commercially available.

Procedure

For preparation and extraction of the experimental material (blood, tissue, *etc.*), see determination of pyruvate (p. 254).

Spectrophotometric measurements

Wavelength: 366 mμ ($\lambda_{max.}$ for AP-DPNH: 363 mμ); glass cuvettes (semi-microcuvettes), light path: 0.5 cm; final volume: 0.4 ml. Light path and final volume may be varied in order to make the test more sensitive. Read experimental and control cuvettes against air or a blank cuvette containing water. Bring buffer and sample to room temperature; pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
0.20 ml buffer (solution I)	0.20 ml. buffer (solution I)
0.10 ml. AP-DPN solution (II)	0.10 ml. AP-DPN solution (II)
sample + water to 0.34 ml.	water to 0.34 ml.

Read the optical density of both cuvettes. If the change in optical density is not greater than 0.001 to 0.002 per 30 seconds, mix

0.06 ml. MDH-suspension (III) (*ca.* 30 μg. protein)

into both cuvettes.

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

The reaction is considered to have stopped (usually after 15—25 min.) when the increase in optical density with time is very small and is the same in the experimental and control cuvette. A control containing all the components of the assay mixture except the enzyme, shows no significant change in optical density with time. The optical density difference between the sample and the control on completion of the reaction minus the optical density difference between sample and control before the start of the reaction with MDH, yields the ΔE required for the calculations.

Since the change in optical density in the control cuvette is limited to that caused by the absorption of the enzyme and the dilution of the test mixture by addition of enzyme, it can usually be omitted thus conserving expensive AP-DPN. In this case the change in optical density caused by the absorption of the enzyme and by dilution of the test mixture, must be obtained by a further addition of the enzyme to the experimental cuvette after completion of the reaction.

Calculations

$$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles malate/cuvette}$$

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

Appendix

Initial velocity of the malate oxidation

The initial velocities of the reactions with 3.3×10^{-5} M AP-DPN or with DPN were compared. In 0.87 M glycine buffer, pH 9.5 and with 16.6 mg. enzyme protein/litre the ratio $V_{DPN} : V_{AP\text{-}DPN}$ was 1 : 1.11.

Michaelis constants

The reaction rates with 3.2×10^{-5} M to 8.15×10^{-3} M L-malate or with 2.5×10^{-5} to 1.0×10^{-3} M AP-DPN were measured in 0.9 M glycine buffer, pH 9.5 at 23.5° C. The constants were calculated according to *Lineweaver* and *Burk* [3]. Under these conditions with 5×10^{-3} M L-malate the K_M for AP-DPN = 6.4×10^{-5} M, and with 7.5×10^{-5} M AP-DPN the K_M for L-malate = 4.1×10^{-4} M.

Equilibrium constant

The equilibrium constant K is defined as

$$K = \frac{[\text{AP-DPNH}] \times [\text{oxaloacetate}] \times [H^+]}{[\text{AP-DPN}^+] \times [\text{malate}]}$$

The malate was determined as described in the above test. AP-DPN was determined by quantitative reduction to AP-DPNH at pH 9.5 with malic dehydrogenase and excess malate (refer to p. 333). The H^+ ion concentration was measured with a glass electrode after the equilibrium was attained. The equilibrium concentrations of AP-DPN and oxalacetate were obtained from the spectrophotometric measurements of the AP-DPNH concentration at equilibrium. The value obtained for K = 2.13×10^{-10} M [4] at 35° C.

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[2] *J. M. Siegel, G. A. Montgomery* and *R. M. Bock*, Arch. Biochem. Biophysics *82*, 288 [1959].
[3] *H. Lineweaver* and *D. Burk*, J. Amer. chem. Soc. *56*, 658 [1934].
[4] *H. D. Söling* and *H. Holzer*, Biochem. Z. *336*, 201 [1962].