Oxaloacetate

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Principle

Malic dehydrogenase (MDH) catalyses the reduction of oxaloacetate with reduced diphosphopyridine nucleotide (DPNH):

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

The equilibrium of the reaction lies far to the right with an equilibrium constant K_c of 2×10^{12} l./mole at 25° C[1]. With a slight excess of DPNH and at about neutral pH the reaction is rapid and oxaloacetate is quantitatively converted to malate. The decrease in optical density at 340 mμ due to the oxidation of DPNH is a measure of the reaction.

Reagents

1. Potassium carbonate, K_2CO_3, A. R.
2. Methyl orange indicator
3. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
4. Triethanolamine hydrochloride
5. Sodium hydroxide, A. R., 2 N
6. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (Titriplex III, Trilon B, Versene)
7. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
8. Malic dehydrogenase, MDH
   from pig heart, suspension in 2.8 M ammonium sulphate solution. Commercial preparation, see p. 988.
9. Potassium dichromate, $K_2Cr_2O_7$, A. R.

Purity of the enzyme preparation

The MDH preparation should have a specific activity of about 2000 units/mg. (according to *Bücher* *[1]) or 36 units/mg. (according to *Racker* *[1]). Contamination by lactic dehydrogenase should not exceed 0.05% of the MDH activity.

Preparation of Solutions

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 *ca.* 7.7 ml. $HClO_4$ (sp. gr. 1.67) to 150 ml. with distilled water.

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

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

IV. Triethanolamine buffer (0.4 M; pH 7.6):
Dissolve 18.6 g. triethanolamine hydrochloride in about 200 ml. distilled water, add 18 ml. 2 N NaOH and 3.7 g. EDTA-$Na_2H_2 \cdot 2H_2O$ and dilute to 250 ml. with distilled water.

V. Reduced diphosphopyridine nucleotide (*ca.* 3×10^{-3} M β-DPNH):
Dissolve 3 mg. DPNH-Na_2 in triethanolamine buffer (solution IV) and make up to 1 ml.

VI. Malic dehydrogenase, MDH (*ca.* 0.3 mg. protein/ml.):
Dilute enzyme suspension containing *ca.* 10 mg. protein/ml. in 2.8 M ammonium sulphate solution with distilled water.

VII. Potassium dichromate (*ca.* 1%):
Dissolve *ca.* 1 g. $K_2Cr_2O_7$ in distilled water and make up to 100 ml.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator at 0–4° C. The DPNH solution in the triethanolamine buffer keeps for 2–3 weeks, but aqueous solutions are less stable. The MDH solution keeps for a long period without loss of activity.

Procedure

Experimental material

Studies on blood and plasma are not very informative because of the extremely low concentration of oxaloacetate (3×10^{-10} moles/g. whole blood)[2]. 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. When only oxaloacetate is to be determined in the tissue extract, extract once and calculate the volume of the extract by assuming a water content in the tissue of 75% (liver, kidney, muscle, heart). To obtain a ratio of volume of extract to tissue weight of 4 : 1, add

6.5 ml. perchloric acid solution

to 2 g. of tissue. If other metabolites are to be determined in the same extract, repeat the extraction with perchloric acid and increase to 6 : 1 the ratio of the volume of extract to the tissue weight.

Method: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 2 g. tissue powder (powdered frozen[3]), mix thoroughly with the glass rod and re-weigh.

Single extraction: Calculate the total volume of perchloric acid required to give a ratio of volume of extract to tissue weight of 4 : 1 (see above) and then add the requisite quantity of the 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.

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[2] *M. Reim* and *H. J. Hohorst*, Klin. Wschr., in press.
[3] *H. J. Hohorst*, *F. H. Kreutz* and *Th. Bücher*, Biochem. Z. *332*, 18 [1959].

Repeated extraction: Mix the suspension of the sample in the perchloric acid already present (in special cases, *e.g.* with muscle or heart tissue, use a homogenizer) and immediately centrifuge at 3000 g. Decant the supernatant, stir the sediment with *ca.* 2 ml. perchloric acid solution (III) + 2 ml. distilled water and re-centrifuge. Combine the supernatants, measure the volume and make up to 6 ml./g. with distilled water.

Neutralization: Pipette 0.01 ml. indicator solution (II) into 4 ml. tissue 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 practically ceased and then add more carbonate solution until the mixture is salmon-pink (*ca.* pH 3.5). Adjust to between pH 5 and 6.5 with carbonate solution (spotting on indicator paper) to ensure that the buffering capacity of triethanolamine in the assay mixture will not be exceeded and to minimize the decomposition of oxaloacetate in the extract (least stable at pH 2 to 3).

Spectrophotometric measurements

Preliminary remarks: The oxaloacetate content of all the tissues so far examined is very low. For example a rat liver extract was found to contain about 10^{-9} moles oxaloacetate/ml. when the extraction method described above was used. The sensitivity of the measuring instrument must therefore be high and the dilution of the extract in the assay mixture must be minimized. Dilution is limited by the absorption and possible turbidity of the extract (due to glycogen). A highly sensitive, recording spectrophotometer is a suitable instrument for the estimations. The method described here was developed for the Beckmann DK-1 spectrophotometer. It is an advantage to record the optical density changes, because then unspecific changes, which are unavoidable with the high concentrations of extract, can be more easily corrected. All solutions must be free from particles and the cuvettes must contain no dust. If necessary, the assay mixture should be filtered through sintered glass.

Method:

Wavelength: 340 mμ; light path: 5 cm.; final volume: 4.52 ml.

Experimental cuvette	*Control cuvette* (light path 1 cm.)
1.5 ml. buffer (solution IV)	2 ml. buffer (solution IV)
3.0 ml. deproteinized extract	

Place the cuvettes in the measurement and reference channels of the instrument, switch the scale of the recorder to 0—100% light transmission. Mix into the experimental cuvette

0.010 ml. DPNH solution (V).

To the control cuvette mix in drop-wise with a glass rod

potassium dichromate solution (VII)

until the light transmission is 95—98%. Before reading, allow for temperature equilibration. Switch the scale to 90—110% light transmission and record the value (T_1) for 3—4 min. at the slowest chart speed. Stir the contents of both cuvettes frequently until either the transmission remains constant (which is usually not the case) or the change with time is constant. Start the reaction by mixing

0.005 ml. malic dehydrogenase solution (VI)

into the experimental cuvette and record the transmission. On completion of the reaction (2 to 3 min.) record the transmission (T_2) for a further 3–4 min. Then obtain the correction for the change in absorption due to the addition of enzyme (T_3) by mixing in a further

0.005 ml. malic dehydrogenase solution (VI).

Calculations

Oxaloacetate reacts quantitatively, therefore the concentration can be calculated from the change in light transmission by means of the equation

$$\log \frac{T_2}{T_1} = \varepsilon \times c \times p \qquad (1)$$

where

T_1 = transmittance before addition of enzyme
T_2 = transmittance after addition of enzyme
ε = extinction coefficient of DPNH at 340 mμ = 6.22 cm.²/μmole
d = light path in cm.
c = concentration in μmoles/ml.

The oxaloacetate content per unit weight of tissue is obtained according to the formula

$$\frac{(\log T_2 - \log T_1) \times \text{dil.}}{\varepsilon \times d} = \mu\text{moles oxaloacetate/g. tissue} \qquad (2)$$

dil. = the total dilution of the tissue in the assay. This is made up of the dilution in the extract (according to the above method *ca.* 4 : 1 or 6 : 1) and the dilution of the extract in the assay mixture (*ca.* 1.5 : 1).

Example

1.79 g. tissue powder (rat liver) were added to 5 ml. perchloric acid. To obtain a ratio of volume of extract to tissue weight of 4 : 1 the perchloric acid was made up to $\frac{1.79}{2} \times 6.5 = 5.84$ ml.; 0.23 ml. carbonate solution were required for neutralization. The ratio of volume of extract to tissue weight is then $\left(5.84 + 0.23 + 1.79 \times \frac{75}{100}\right) : 1.79 = 4.16 : 1$. The amount of neutralized extract taken for the assay was 3 ml. from which it follows that, with an assay volume of 4.52 ml., the total dilution of the tissue is

$$\text{dil.} = 4.16 \times \frac{4.52}{3} = 6.29$$

At 340 mμ and with a scale range of 90–110% transmission the following values were measured:
before addition of the enzyme: $T_1 = 0.944$ (94.4%); $\log T_1 = \bar{1}.9750$
after addition of the enzyme: $T_2 = 1.008$ (100.8%) $\log T_2 = 0.0035$

$$\log T_2 - \log T_1 = 0.0285.$$

Therefore according to equation (2) the oxaloacetate content of the tissue

$$c' = \frac{0.0285 \times 6.29}{6.22 \times 5} = 5.7 \times 10^{-3}\ \mu\text{moles/g.}$$

Other Determinations

Other metabolites can be determined in the same assay mixture by the addition of DPNH and the corresponding enzymes: *e.g.* dihydroxyacetone phosphate with glycerol-1-phosphate dehydrogenase (α-glycerophosphate dehydrogenase), fructose-1,6-diphosphate by the addition of aldolase and triosephosphate isomerase (refer to p. 246) and pyruvate with lactic dehydrogenase (refer to p. 253).

Sources of Error

Since the measurements must be carried out with the maximum amplification of the spectrophotometer, interference, particularly that due to the greater background noise (>1%), may increase. In certain cases with very turbid extracts (liver glycogen) the ratio of the volume of extract to the assay volume must be reduced; occasionally high speed centrifugation (5 min. at 50000 g) proves effective in removing the turbidity. It is recommended that a UV filter be placed in the light path of the spectrophotometer because of the high slit widths required.

DPNH should only be added to the extract-buffer mixture just before the start of the assay, since otherwise a loss of oxaloacetate occurs. This is caused by the slight malic dehydrogenase activity, which even after the deproteinization with perchloric acid is still present in the extract, and which in the presence of the added DPNH reduces oxaloacetate at a measurable rate[4]. In contrast, the spontaneous decomposition of oxaloacetate in the extract (maximal at pH 2—3) is not important, as long as the pH of the extract is adjusted to between 5 and 6 and the extract is stored in a refrigerator. However, the extract should not be frozen and the determination should be carried out as soon as possible after the preparation of the extract.

The enzyme preparation should not contain lactic dehydrogenase, otherwise the larger amounts of pyruvate usually present in extracts will interfere.

Specificity

The reaction of other α-oxo-dicarboxylic acids[5] with malic dehydrogenase need not be considered when estimating oxaloacetate in tissue extracts.

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[4] *H. J. Hohorst* and *M. Reim*, unpublished.

[5] *D. D. Davies* and *E. Kun*, Biochem. J. *66*, 307 [1957].