Fumarate

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Principle

The most convenient method for the determination of fumarate in biological material is based on its conversion to L-lactate by the action of fumarase (1), malic enzyme (2) and lactic dehydrogenase (3)[1,2]:

(1) $\text{Fumarate} + H_2O \rightleftharpoons \text{L-malate}$

(2) $\text{L-Malate} + DPN^+ \rightleftharpoons \text{pyruvate} + CO_2 + DPNH + H^+$

(3) $\text{Pyruvate} + DPNH + H^+ \rightleftharpoons \text{L-lactate} + DPN^+$

The balance equation is:

(4) $\text{Fumarate} + H_2O \rightleftharpoons \text{L-lactate} + CO_2$

Under suitable conditions the over-all reaction (4) proceeds quantitatively from left to right. For each mole of fumarate 1 mole of CO_2 is formed and the reaction can therefore be followed manometrically.

If the sample contains L-malate, the sum of the fumarate + L-malate is obtained. L-Malate can be determined separately by carrying out a second assay with the omission of fumarase. The difference in the two assays gives the fumarate content of the sample.

Reagents

1. Potassium acetate, A. R.
2. Acetic acid, A. R.
3. Potassium hydroxide, A. R., 0.1 N
4. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
5. Manganous chloride, $MnCl_24 \cdot H_2O$, A. R.
6. Fumaric acid, A. R.
7. Fumarase
   from pig heart[3] (the authors have had no experience with the preparation described by[4]).
8. Malic enzyme-lactic dehydrogenase
   A homogenate of lyophilized *Lactobacillus arabinosus* cells is used. For growth of the bacteria, see p. 349.

Purity of the enzyme preparations

The specific activity*) of the fumarase preparation should be at least 50 *Massey* units. It is sufficient to carry the purification according to[3] only through the calcium phosphate gel stage. The enzyme should be stored in ammonium sulphate solution (60% saturation) at 0°C. The solution keeps for years.

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*) A unit according to *Massey*[3] is the amount of enzyme contained in a 3 ml. assay mixture, which changes the optical density at 300 mμ by 0.010 in 1 min. at pH 7.3. The amount of enzyme is determined by the optical density E_{277} at 277 mμ (light path: 1 cm.). Specific activity = units/ml. × E_{277}.

1) *M. L. Blanchard, S. Korkes, A. del Campillo* and *S. Ochoa*, J. biol. Chemistry *187*, 875 [1950].
2) *S. Korkes, A. del Campillo* and *S. Ochoa*, J. biol. Chemistry *187*, 891 [1950].
3) *V. Massey* in *S. Colowick* and *N. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 729.
4) *C. Frieden, R. M. Bock* and *R. A. Alberty*, J. Amer. chem. Soc. *76*, 2482 [1954].

Lyophilized *L.arabinosus* cells usually contain so little fumarase, that fumarate is not converted to L-malate and therefore no CO_2 is formed from fumarate. If this is not the case, then a malic enzyme-lactic dehydrogenase preparation purified according to *Ochoa*[5] must be used and DPN must be added to the assay mixture. The advantage of working with the lyophilized cells lies in the ease of preparation and preservation, and the fact that addition of DPN is not necessary.

Preparation of Solutions

I. Potassium acetate buffer (*ca.* 2 M; pH 5.5):
Titrate *ca.* 2 M potassium acetate solution (196 g. $KC_2H_3O_2$/1000 ml.) with *ca.* 2 N acetic acid (115 ml. acetic acid diluted to 1000 ml.) or with 2 N HCl to pH 5.5 (pH-meter).

II. Manganous chloride (*ca.* 0.045 M):
Dissolve 0.89 g. $MnCl_2 \cdot 4H_2O$ in distilled water and make up to 100 ml.

III. Fumarate standard solution (0.050 M; pH 5.5):
Dissolve 0.58 g. fumaric acid in 50 ml. distilled water, adjust to pH 5.5 (pH-meter) with 0.1 N KOH and dilute to 100 ml. with distilled water.

IV. Fumarase (250 units*)/ml.):
Dilute the solution or the suspension of the enzyme preparation in ammonium sulphate solution with phosphate buffer (*ca.* 0.01 M; pH 6; 1.4 g. KH_2PO_4 adjusted with 0.1 N KOH to pH 6 and diluted with distilled water to 1000 ml.).

V. *L. arabinosus* homogenate
Homogenize 100 mg. lyophilized cells (see p. 349) in 1 ml. distilled water in a glass or glass-Teflon homogenizer.

Stability of the solutions

The acetate buffer keeps in a refrigerator for several months. The fumarate standard solution should be stored frozen and in this state it is stable for months. The fumarase solution and the *L. arabinosus* homogenate should be prepared freshly each day.

Procedure

Experimental material

Deproteinize tissue samples with $HClO_4$ and remove the excess perchlorate by neutralization with K_2CO_3 or KOH solution (see determination of succinate, p. 342). Then adjust the pH of the sample to 5.5.

Manometric measurements

Preliminary remarks: Korkes[6] recommended a pH of 6.0 for the determination of fumarate + L-malate. We prefer pH 5.5 for the following reason: if the assay is performed at pH 6.0, double side-arm Warburg vessels are required. At the end of the reaction acid must be tipped into the main compartment from the second side-arm to liberate the retained CO_2. As a result this method has large blanks unless CO_2-free reagents are used. However, at pH 5.5 the CO_2 retention is negligible and the recovery of added fumarate is 96 to 100%. On the other hand, pH 5.5 is unfavourable for the action of fumarase and the malic enzyme, and

*) Refer to the footnote on p. 346.

5) *S. Ochoa* in *S. Colowick* and *N. Kaplan*: Methods in Enzymology. Academic Press, New York, 1955, Vol. I, p. 739.

6) *S. Korkes* in *S. Colowick* and *N. Kaplan*: Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 435.

therefore a sufficient excess of the two enzymes must be used in order to bring the reaction to completion within a reasonable period.

Method: Measurements with the Warburg apparatus; temperature: 38°C; gas phase: air. Five 15 to 25 ml. Warburg vessels are required (2 for a duplicate assay on the unknown sample, 2 for the standard fumarate solution and 1 for the fumarate-free control).

Pipette into the *side-arms* of all the vessels:

0.15 ml. acetate buffer (solution I)
0.40 ml. *L. arabinosus* homogenate (V).

Pipette into the *main compartment* of all the vessels:

0.6 ml. acetate buffer (solution I)
0.1 ml. $MnCl_2$ solution (II).

To the experimental vessels add:

sample
0.1 ml. fumarase solution (III)
distilled water to 2.45 ml.

To the standard vessels add:

0.1 ml fumarase solution (IV)
0.1 and 0.2 ml. fumarate standard solution (III)
distilled water to 2.45 ml.

To the control vessel add:

distilled water to 2.45 ml.

Equilibrate for 10 min. at 38°C, close the taps and read the manometers. Tip the contents of the side-arm into the main compartment and continue to read the manometers until the rate of increase in pressure in the experimental and standard vessels is the same as that in the control. If the experimental vessel contains 1—10 μmoles fumarate, the reaction should be complete in 15—25 min. The amount of CO_2 evolved in the control is about 5—7 μl. (endogenous CO_2 production from the lyophilized cells).

To determine fumarate instead of the sum of fumarate + L-malate prepare a further two vessels containing no fumarase for each sample. The resulting CO_2 production corresponds to the L-malate content of the sample, while the CO_2 evolved in the vessels containing fumarase corresponds to the sum of the fumarate + L-malate. The difference in the two volumes of CO_2 corresponds to the fumarate content of the sample.

Calculations

The following formula is used for the calculations (refer also to p. 40):

$$\frac{(\mu l.\ CO_2 \text{ produced in experimental vessel}) - (\mu l.\ CO_2 \text{ produced in control vessel})}{22.4} = \mu\text{moles (fumarate + L-malate)/experimental vessel}$$

Sensitivity

The sensitivity of the method depends on the type of Warburg apparatus used. With 15 to 30 ml. vessels the lower limit for the determination is 1 μmole (fumarate + L-malate), with 5 ml. vessels it is about 0.3 μmoles. If greater sensitivity is required without recourse to special micro-apparatus,

fumarate can be reduced to succinate[6] by Zn in acid solution and the succinate can be determined enzymatically (see p. 340). The succinate already present in the sample must be determined separately by omission of the reduction step and this value must be subtracted from the sum of the succinate + fumarate obtained after the reduction with Zn. This method is unsuitable if the concentration of succinate in the sample is much greater than that of fumarate.

Sources of Error

Any pyruvate present in the sample is decarboxylated[6] and therefore interferes. If the presence of pyruvate is suspected, then semicarbazide, which prevents the decarboxylation of pyruvate, should be added to the reaction mixture to give a final concentration of 0.02 M (pH 5.5)[6].

The presence in the sample of substances which inhibit the enzymes used can easily be detected by adding a known amount of the fumarate standard solution to the sample and measuring its recovery, which should be 96–100%.

Appendix

Preparation of Lyophilized L. arabinosus Cells

Reagents

Difco yeast extract *)
Difco nutrient broth *)
Glucose
DL-Malic acid
Sodium acetate·$3H_2O$
Dipotassium hydrogen phosphate, K_2HPO_4
Sodium chloride
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$
Manganous sulphate, $MnSO_4 \cdot 4H_2O$
Ferrous sulphate, $FeSO_4 \cdot 7H_2O$

Preparation of the salt solution

Dissolve 2 mg. NaCl + 40 mg. $MgSO_4 \cdot 7H_2O$ + 2 mg. $MnSO_4 \cdot 4H_2O$ + 2 mg. $FeSO_4 \cdot 7H_2O$ in distilled water and make up to 1000 ml. Sterilize by filtering through a Seitz filter.

Procedure

Strain of bacteria: Lactobacillus arabinosus 17-5 (ATCC **) 8014).

Growth medium: For stab cultures: 1% yeast extract, 1% glucose and 1.5% agar[6]. Transfer every 3–4 weeks, incubate for 24 hours at 30°C.

For subculture: 1% yeast extract and 1% glucose. After inoculation incubate at 30°C.

For large scale culture: 2% glucose, 2% DL-malic acid, 1% yeast extract, 1% nutrient broth, 1% Na acetate·$3H_2O$, 0.1% K_2HPO_4 and 5 ml. salt solution. Sterilize the medium without the glucose and salt solution. Sterilize the glucose separately as a 20% solution and add aseptically to the rest of the medium. Add the sterile salt solution (Seitz filtration) aseptically to the rest of the medium.

Growth of the bacteria: Inoculate the growth medium with part of the subculture (1–5% v/v) and incubate for 24 hours at 30°C (stir continuously by blowing in N_2 through a coarse sintered glass bubbler). Centrifuge off the cells and wash with distilled water until the supernatant is clear and colourless. Lyophilize the cells. From a 10000 ml. culture the yield is about 9 g. lyophilized cells. These keep for several months in a desiccator below 0°C.

*) Difco Laboratories, Inc., Detroit 1, Michigan, USA.
**) American Type Culture Collection, 2112 M Street, North, Washington 7, D. C., USA.