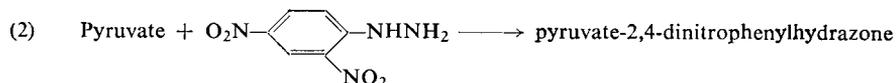
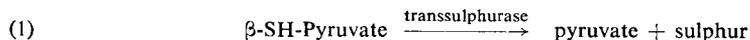


β -Mercaptopyruvate

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Principle

β -Mercaptopyruvate is split by transsulphurase according to equation (1) to give sulphur and pyruvate¹⁻³). The latter is determined colorimetrically as the 2,4-dinitrophenylhydrazone.



It is not possible to determine the pyruvate formed enzymatically with lactic dehydrogenase (LDH, see p. 253) because β -mercaptopyruvate also reacts with LDH⁴). Theoretically any residual mercaptopyruvate could be removed with cadmium acetate, but excess Cd^{2+} inhibits LDH.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4 , A. R.
2. Cadmium acetate $\cdot 3\text{H}_2\text{O}$, A. R.
3. 2-Mercaptoethanol, A. R.
4. 2,4-Dinitrophenylhydrazine
5. Hydrochloric acid, 2 N
6. Toluene, A. R.
7. Sodium carbonate, $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$
8. Sodium hydroxide, 1.5 N
9. Sodium pyruvate *)
crystalline, prepared according to⁵); commercial preparation, see p. 1027.
10. Transsulphurase
isolation, see p. 265.

Purity of the enzyme preparation

A preparation having a specific activity of 40—50 units **)/mg. is satisfactory. The contaminating enzyme activities do not interfere with the determination.

Preparation of Solutions

I. Phosphate buffer (0.02 M; pH 7.45):

Dissolve 0.348 g. K_2HPO_4 in distilled water, adjust to pH 7.45 with 2 N HCl and make up to 100 ml.

*) The purity of the pyruvate preparation must be determined (Pyruvate Determination, see p. 253).

***) A preparation has a specific activity of 1 unit, when 1 mg. forms 1 μ mole pyruvate from β -mercaptopyruvate at 30° C in 10 min., under the conditions stated in this chapter. The reaction is linear for the first 20 min. and the rate is proportional to the amount of enzyme.

¹) E. Kun and D. W. Fanshier, *Biochim. biophysica Acta* 27, 659 [1958].

²) E. Kun and D. W. Fanshier, *Biochim. biophysica Acta* 32, 338 [1959].

³) E. Kun and D. W. Fanshier, *Biochim. biophysica Acta* 33, 26 [1959].

⁴) E. Kun, *Biochim. biophysica Acta* 25, 135 [1957].

⁵) *Biochemical Preparations*. Wiley, New York 1952, Vol. II, p. 22.

- II. Cadmium acetate solution (saturated):
Dissolve *ca.* 160 g. cadmium acetate·3H₂O in *ca.* 100 ml. hot distilled water, allow to cool and use the supernatant.
- III. 2-Mercaptoethanol:
Use undiluted.
- IV. 2,4-Dinitrophenylhydrazine solution (0.1%):
Dissolve 100 mg. 2,4-dinitrophenylhydrazine in 100 ml. 2 N HCl. Keep in a dark bottle at 4°C.
- V. Na₂CO₃ solution (10% w/v Na₂CO₃):
Dissolve 27 g. Na₂CO₃·10H₂O in distilled water and make up to 100 ml.
- VI. Sodium hydroxide (1.5 N):
Dissolve 6 g. NaOH in distilled water and make up to 100 ml.
- VII. Pyruvate standard solution (0.02 M):
Dissolve 22 mg. (or more according to the purity of the preparation) sodium pyruvate in distilled water and make up to 10 ml.
- VIII. Transsulphurase solution (*ca.* 10 mg. protein/ml.):
Dissolve 10 mg. dry preparation in 1 ml. phosphate buffer (solution I).

Stability of the solutions

Keep NaOH and Na₂CO₃ solutions well stoppered. Prevent bacterial growth in the phosphate buffer by storing at 4°C. Prepare the 2,4-dinitrophenylhydrazine solution freshly each week and store at 4°C. The enzyme solution keeps at -15°C for 3-6 weeks.

Procedure

Experimental material

β-Mercaptopyruvate is a labile compound. For preparation of the ammonium salt, see^{4,6)}. This salt is stable indefinitely in a desiccator at 4°C.

Pyruvate standard curve

Prepare two standards of 10 and 20 μmoles pyruvate:

- 0.5 ml. solution VII + 0.5 ml. distilled water or
- 1.0 ml. solution VII.

Add

- 2.0 ml. 2,4-dinitrophenylhydrazine solution (IV)

and proceed as described under "Spectrophotometric measurements". Plot a standard curve with the values (optical density at 520 mμ against amount of pyruvate).

Incubation mixture

A reagent blank with water instead of enzyme solution is prepared for each determination. Pipette successively into a test tube standing in ice-water:

- 1.5 ml. phosphate buffer (solution I)
- 0.5 ml. sample (50 μmoles freshly dissolved β-mercaptopyruvate)
- 0.1 ml. 2-mercaptoethanol (solution III)
- 0.3 ml. distilled water
- 0.1 ml. transsulphurase solution (VIII).

⁶⁾ W. D. Kumler and E. Kun, Biochim. biophysica Acta 27, 464 [1957].

Start the reaction by placing the tubes in a water bath at 30°C. After 10 min. stop the reaction by the addition of

1 ml. saturated cadmium acetate solution (II).

Shake vigorously, allow to stand 10 min. at room temperature, centrifuge (3000 r.p.m.) and decant supernatant fluid from the precipitate.

Spectrophotometric measurements

Into a clean test-tube pipette:

1 ml. supernatant

2 ml. 2,4-dinitrophenylhydrazine solution (IV).

Incubate for 20 min. at 30°C. Extract the pyruvate dinitrophenylhydrazone with

3 ml. toluene

in a small separating funnel; extract the toluene layer with

5 ml. carbonate solution (V)

and filter the carbonate extract. For colorimetric measurements pipette into a clean test-tube:

3 ml. carbonate extract

5 ml. 1.5 N NaOH (solution VI).

After 5 min. measure the optical density at 520 m μ .

Calculations

Obtain from the standard curve the pyruvate value corresponding to the measured optical density. According to equation (1) 1 μ mole pyruvate corresponds to 1 μ mole β -mercaptopyruvate. To correct for the dilution of the 0.5 ml. sample in the above procedure the pyruvate value must be multiplied by 7 to obtain μ moles β -mercaptopyruvate/ml. sample.

Sources of Error

Transsulphurase is the most specific reagent for β -mercaptopyruvate; K_s for β -mercaptopyruvate²⁾ is 2.7×10^{-3} . Lactic dehydrogenase (LDH) reduces β -mercaptopyruvate in the presence of DPNH to β -mercaptolactate. K_s of LDH from heart muscle for β -mercaptopyruvate is 8.2×10^{-4} compared to 5.4×10^{-5} with pyruvate⁴⁾. Pyruvic carboxylase decarboxylates β -mercaptopyruvate to give CO₂ and mercaptoacetaldehyde⁷⁾.

Appendix

Isolation of transsulphurase¹⁻³⁾

The preparation of the enzyme includes the following steps: preparation of an acetone powder from rat liver (stable for 3–5 months at -30°C); extraction of the dry powder with phosphate buffer; fractionation with ammonium sulphate; lyophilization of the protein precipitated between 0.2 and 0.4 g. (NH₄)₂SO₄/ml. The lyophilized preparation is stable for 3–5 months at -30°C . Its specific activity is 40–50 units (for definition, see footnote on p. 263). The enzyme is highly sensitive to the inhibitory action of metal chelating agents⁸⁾. For further properties see⁹⁾.

⁷⁾ E. Kun and H. G. Williams-Ashman, *Experientia* 18, 261 [1962].

⁸⁾ E. Kun and D. W. Fanshier, *Biochim. Biophysica Acta* 48, 187 [1961].

⁹⁾ D. W. Fanshier and E. Kun, *Biochim. Biophysica Acta* 58, 266 [1962].