Orotic acid

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Principle

Dihydroorotic dehydrogenase from *Zymobacterium oroticum* catalyses the reduction of orotic acid by reduced diphosphopyridine nucleotide (DPNH)[1]:

(1) $$\text{Orotate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{dihydroorotate} + \text{DPN}^+$$

The equilibrium of the reaction lies in favour of dihydroorotic acid; the equilibrium constant[2] K is 2.4×10^9 [l./mole]. The enzyme has a turnover number based on the flavin content of the order of 1200[2a]. The rate of reduction of orotic acid is maximal at about pH 6.5. With excess DPNH the reaction proceeds at a sufficient rate, and orotic acid is nearly quantitatively reduced. However, under aerobic conditions more DPNH is oxidized than that expected from the orotic acid content of the sample[1,3]. Consequently the reduction of orotic acid is determined directly by the decrease in absorption at 282 mμ[1]. DPNH and DPN are isosbestic at this wavelength. The reaction requires the presence of cysteine which is slowly oxidized causing an increased absorption at 282 mμ. Thus it is necessary to set up a control cuvette containing all the reagents with the exception of the dihydroorotic dehydrogenase. The enzymic reaction is considered at an end when the optical density of the experimental cuvette begins to increase at the same rate as that of the control cuvette.

Reagents

1. Sodium dihydrogen phosphate, $NaH_2PO_4 \cdot H_2O$
2. Disodium hydrogen phosphate, Na_2HPO_4 or $Na_2HPO_4 \cdot 7H_2O$
3. Cysteine hydrochloride
4. Sodium hydroxide, 1 N
5. Orotic acid
   recrystallized twice from water; commercial preparation, see p. 1023.
6. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
7. Dihydroorotic dehydrogenase
   crystalline from *Zymobacterium oroticum*[4]. For isolation, see p. 511.

Preparation of Solutions

I. Phosphate buffer (0.2 M; pH 5.8):
Mix 92 ml. 0.2 M NaH_2PO_4 solution (2.76 g. $NaH_2PO_4 \cdot H_2O$ contained in 100 ml.) and 8 ml. 0.2 M Na_2HPO_4 solution (2.84 g. Na_2HPO_4 or 5.36 g. $NaHPO_4 \cdot 7\,H_2O$ contained in 100 ml.).

II. Phosphate buffer (1 M; pH 6.2):
Mix 81.5 ml. 1 M NaH_2PO_4 solution (13.8 g. $NaH_2PO_4 \cdot H_2O$ contained in 100 ml.) and 18.5 ml. 1 M Na_2HPO_4 solution (14.2 g. Na_2HPO_4 or 26.81 g. $Na_2HPO_4 \cdot 7\,H_2O$ contained in 100 ml.).

III. Cysteine hydrochloride (0.1 M):
Just before use dissolve 79 mg. cysteine hydrochloride in distilled water, adjust to pH 6.2 with 1 N NaOH and make up to 5 ml.

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[1] *I. Lieberman* and *A. Kornberg*, Biochim. biophysica Acta *12*, 223 [1953].
[2] *G. Krakow* and *B. Vennesland*, J. biol. Chemistry *236*, 142 [1961].
[2a] *G. Krakow* and *B. Vennesland*, unpublished observations.
[3] *H. C. Friedmann* and *B. Vennesland*, J. biol. Chemistry *233*, 1398 [1958].
[4] *H. C. Friedmann* and *B. Vennesland*, J. biol. Chemistry *235*, 1526 [1960].

IV. Sodium orotate (*ca.* 0.02 M):
To a suspension of 0.312 g. orotic acid in distilled water, add 4 ml. 1 N NaOH and dilute with distilled water to 100 ml.

V. Reduced diphosphopyridine nucleotide (*ca.* 0.003 M β-DPNH):
Dissolve 8.4 mg DPNH-Na_2 in distilled water and dilute to 3 ml.

VI. Dihydroorotic dehydrogenase:
Extract the crystalline enzyme with ice-cold 0.2 M phosphate buffer (solution I), remove the slight amount of white insoluble matter by centrifuging. Dilute the supernatant with cold 0.2 M phosphate buffer (solution I) so that in the enzyme standardization procedure a decrease in optical density of about 0.2 in 3 minutes is obtained (refer to "Procedure").

Stability of the solutions

Store solutions I and II at room temperature, IV and VI at 4°C, V at −15°C and prepare III just before use. A diluted solution of the enzyme (VI) quickly loses its activity above about 20°C.

Procedure

Preliminary remarks: The method for the determination of orotic acid described here has only been tested with for pure sodium orotate solutions. Attempts to estimate orotic acid in tissue extracts after purification, for example, by chromatographic separation on paper, on Dowex 50[5] or on Dowex 2[5a] columns, have not been made.

Enzyme standardization

Wavelength: 340 mμ; light path: 1 cm.; final volume: 3 ml.

Read against a blank cuvette containing distilled water. Pipette the solutions in the given order into the cuvette:

0.4 ml. buffer (solution II)
0.2 ml. cysteine hydrochloride solution (III)
0.3 ml. orotate solution (IV)
1.0 ml. distilled water
enzyme solution (VI) and
distilled water to give 2.9 ml.

Mix, allow to stand for 10 min. at 20°C, add

0.1 ml. DPNH solution (V),

mix and take readings at 30second intervals as soon as possible. Use for the assay the amount of enzyme which gives a decrease in optical density of about 0.2 in 3 min.

Spectrophotometric measurements

Wavelength: 282 mμ; light path: 1 cm.; final volume: 3 ml.

Read against a blank cuvette containing distilled water. Special care should be taken that the experimental and control cuvettes contain exactly the same volumes of orotate, cysteine, and DPNH solutions.

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[5] *R. A. Yates* and *A. B. Pardee*, J. biol. Chemistry *221*, 743 [1956].
[5a] *P. Reichard*, J. biol. Chemistry *197*, 391 [1952].

Pipette the solutions in the given order:

Experimental cuvette	*Control cuvette*
0.4 ml. buffer (solution II)	0.4 ml. buffer (solution II)
0.2 ml. cysteine solution (III)	0.2 ml. cysteine solution (III)
sample (containing 0.03—0.12 μmoles orotate)	sample (as experimental cuvette)
1.0 ml. distilled water	distilled water to give 2.9 ml.
enzyme solution (VI) (amount according to standardization)	
distilled water to give 2.9 ml.	

Mix, allow to stand for 10 min. at 20°C, add

0.1 ml. DPNH solution (V)

to the experimental and control cuvettes. Mix and read at 30 second or 1 minute intervals, until the optical density of the experimental cuvette begins to increase at the same rate as that of the control cuvette. This point is reached shortly after the optical density of the experimental cuvette has ceased to decrease. The reaction should be complete in 10—15 min.

Instead of reading the optical density of the experimental and control cuvettes separately against water, it is possible to measure the optical density of the control cuvette against the experimental cuvette. In this case the reaction is complete as soon as the optical density reaches a constant value.

Calculations

Under the conditions of the assay described here orotic acid has an extinction coefficient[6] ε_{282} of 7.5 cm.^{2}/μmole. Therefore the optical density change occurring during the experiment is multiplied by 0.4 in order to obtain the orotic acid content of the experimental cuvette in μmoles orotate/3 ml. If the experimental and control cuvettes are read against water, then the optical density change is the difference [(final reading of control cuvette) − (final reading of experimental cuvette)]. The contribution of the enzyme in the experimental cuvette to the absorption at 282 mμ can be ignored.

In tests with 0.04, 0.08 and 0.12 μmoles orotic acid the measured optical density changes agreed with the calculated value by 94 ±1%, 95 ±1% and 96 ±1% respectively. 0.03 μmoles Orotic acid gave 91% of the expected optical density change. The limit of sensitivity of the method is 0.008 μmoles orotate/3 ml.

Sources of Error

In addition to orotate, dihydroorotic dehydrogenase also reduces 5-fluoroorotate in the presence of DPNH. 5-Fluoroorotate has the same Michaelis constant (about 1.3×10^{-4} M), but a greater maximum velocity[3]. No other pyrimidine substrates are known. Uracil, cytosine, 5-methylcytosine and thymine are not active as substrates or inhibitors[1]. 2×10^{-3} M 5-methyl orotate inhibits the reduction of orotic acid about 50%. 0.2 M NaCl and 0.2 M sodium phosphate (pH 6.5) inhibit about 55% and 20% respectively.

[6] *D. Shugar* and *J. J. Fox*, Biochim. biophysica Acta *9*, 199 [1952].

Appendix

Dihydroorotic dehydrogenase

The enzyme is obtained as a flavoglobulin from cell-free extracts of *Z. oroticum*. The purification[4] involves the following steps: Repeated treatment with protamine sulphate to remove nucleic acids, interspersed with ammonium sulphate fractionation and precipitation by dialysis. The nucleic acid-free enzyme crystallizes from 0.2 M NaH_2PO_4 solution in the form of fine, blunt, orange-yellow needles. It contains one mole each of FMN and FAD/62,000 g. protein. The yellow colour rapidly and almost completely disappears on treatment with excess DPNH or dithionite. On treatment with excess dihydroorotic acid the colour disappears more slowly and less completely, and requires cysteine and anaerobiosis. Pre-incubation of the enzyme with cysteine is necessary for maximal activity.