Reduced Triphosphopyridine Nucleotide (TPNH)

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Principle

The glutamic dehydrogenase (GlDH) catalysed reduction of α-oxoglutarate can be used for the determination of TPNH:

(1) α-Oxoglutarate + NH_4^+ + TPNH $\rightleftharpoons$ glutamate + TPN^+

The equilibrium constant $K = \frac{[\text{glutamate}] \times [TPN^+]}{[\alpha\text{-oxoglutarate}] \times [NH_4^+] \times [TPNH]}$

is 10^6 [l./mole] at 25° C. The equilibrium therefore lies greatly in favour of TPN formation. Glutamic dehydrogenase reacts at the same rate with either TPNH or DPNH. For the determination of TPNH, the DPNH must first be oxidized with lactic dehydrogenase and pyruvate (p. 253).

Reagents

All the reagents required for the determination of DPNH (see p. 531). In addition:

9. Sodium hydroxide, A. R., 3 N
10. α-Oxoglutaric acid
    commercial preparation, see p. 1024.
11. Ammonium chloride, A. R.
12. Glutamic dehydrogenase, GlDH
    crystalline, from liver, suspension in 2 M ammonium sulphate solution. Commercial preparation, see p. 978.

    Purity of the enzyme preparation
    The GlDH should have a specific activity of at least 3500 units/mg. according to *Bücher*[1] or 60 units/mg. according to *Racker*[2] (measured with α-oxoglutarate and DPNH).

Preparation of Solutions

All the solutions required for the determination of DPNH (see p. 531). In addition:

VIII. α-Oxoglutarate (0.5 M):
Dissolve 0.73 g. α-oxoglutaric acid in 3 ml. doubly distilled water, adjust to pH 7 with 3 N NaOH and dilute to 10 ml. with doubly distilled water.

IX. Ammonium chloride (1 M):
Dissolve 0.54 g. NH_4Cl in doubly distilled water and make up to 10 ml.

X. Glutamic dehydrogenase, GlDH (20 mg. protein/ml.):
Dilute the stock solution with 2 M ammonium sulphate solution.

Stability of the solutions

All the solutions are stable for several weeks if stored, stoppered, in a refrigerator.

Procedure

Preliminary remarks: Extract TPNH from samples as described for DPNH (refer to p. 532). Like DPNH, TPNH is only completely extracted with alcoholic KOH.

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[1] *G. Beisenherz, H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].

[2] *J. Cooper, P. A. Srere, M. Tabachnik* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].

Spectrophotometric measurements

Wavelength: 340 or 334 mμ (not 366 mμ); light path: 2 cm. or 4 cm.; final volume: 2.025 or 4.050 ml. (with a 4 cm. light path: 4.050 ml.; in this case take double the volumes of all the solutions); room temperature. Measure against a control cuvette containing dilute potassium dichromate solution to compensate for the colour and turbidity of the extracts.

Pipette successively into the cuvette:

2.000 or 4.000 ml. extract
0.005 or 0.010 ml. pyruvate solution (VI)
0.005 or 0.010 ml. α-oxoglutarate solution (VIII)
0.005 or 0.010 ml. ammonium chloride solution (IX).

Mix and read the optical density E_1. Mix in

0.005 ml. or 0.010 ml. LDH suspension (VII)

and after about 3 min. read the optical density E_2. Mix in

0.005 or 0.010 ml. GlDH suspension (X)

and after about 10 min. read the final optical density E_3.

The TPNH content is obtained from the $\Delta E_{TPNH} = E_2 - E_3$. The formula for the calculations is as for DPN, see p. 530.

Sources of Error and Specificity

Because of the small amounts of TPNH in biological material accurate measurements require sensitive instruments. TPNH is destroyed in alkaline extracts more easily than DPNH, especially on heating (keep exactly to the time of heating). After preliminary oxidation of DPNH the assay method is specific for TPNH.

Other Methods for the Determination of TPNH

The oxidation of TPNH by oxidized glutathione and glutathione reductase can also be used for the determination of TPNH[3,4]:

(2) $$\text{Glutathione} + \text{TPNH} + H^+ \rightleftharpoons 2\ \text{glutathione} + TPN^+$$

The equilibrium is greatly in favour of TPN formation. The enzyme is absolutely specific for TPNH, therefore TPNH can be determined *in the presence* of DPNH. However, the specific activity of this preparation is low in comparison to that of the glutamic dehydrogenase.

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[3] *M. M. Ciotti* and *N. O. Kaplan* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology Academic Press, New York 1957, Vol. III, p. 890.

[4] *M. Klingenberg* and *W. Slenczka*, Biochem. Z. *331*, 486 [1959].