Reduced Diphosphopyridine Nucleotide (DPNH)

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Principle

In principle, any DPN-specific dehydrogenase reaction in which DPNH is quantitatively oxidized can be used for the determination of this compound. The oxidation of DPNH by pyruvate, which is catalysed by lactic dehydrogenase (LDH), is the basis of the following method:

(1) $$DPNH + H^+ + \text{pyruvate} \overset{LDH}{\rightleftharpoons} DPN^+ + \text{lactate}$$

The equilibrium constant $K_{pH} = \frac{K}{[H^+]} = \frac{[\text{pyruvate}] \times [DPNH]}{[\text{lactate}] \times [DPN^+]}$ is $K_7 = 2.9 \times 10^{-5}$ (at pH 7 and 25° C).

Therefore the equilibrium of the reaction lies far in favour of DPN, so that even with only a relatively small excess of substrate a quantitative oxidation of DPNH is obtained.

Reagents

1. Ethanol, absolute, A. R.
2. Potassium hydroxide, A. R.
3. Triethanolamine hydrochloride
4. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
5. Dipotassium hydrogen phosphate, K_2HPO_4, A. R.
6. Ammonium sulphate, A. R.
7. Sodium pyruvate
   commercial preparation, see p. 1027.
8. Lactic dehydrogenase, LDH
   crystalline, from skeletal muscle, suspension in 2.1 M ammonium sulphate solution. Commercial preparation, see p. 986.

   Purity of the enzyme preparation
   The LDH preparation should have a specific activity of at least 15000 units/mg. according to *Bücher*[1], corresponding to *ca.* 270 units/mg. according to *Racker*[2] (definition of units, see p. 32 and 33). It should be free from TPN-specific dehydrogenases.

Preparation of Solutions

I. Alcoholic potassium hydroxide (0.5 N):
   Dissolve 2.8 g. KOH in a mixture of equal parts by volume of ethanol and doubly distilled water and make up to 100 ml. with this mixture.

II. Alcoholic potassium hydroxide (1.0 N):
   Dissolve 5.6 g. KOH in 100 ml. ethanol.

III. Triethanolamine-HCl-phosphate mixture (0.5 M triethanolamine; 0.4 M KH_2PO_4; 0.1 M K_2HPO_4):
   Dissolve 9.30 g. triethanolamine hydrochloride, 5.44 g. KH_2PO_4 and 1.74 K_2HPO_4 in doubly distilled water and make up to 100 ml.

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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].

IV. Triethanolamine-HCl (1 M):
Dissolve 18.6 g. triethanolamine hydrochloride in doubly distilled water and make up to 100 ml.

V. Saturated ammonium sulphate solution:
Suspend *ca.* 80 g. $(NH_4)_2SO_4$ in 100 ml. doubly distilled water at room temperature and allow to stand for several hours with occasional stirring. Do not remove undissolved crystals.

VI. Pyruvate (1 M):
Dissolve 1.10 g. sodium pyruvate in doubly distilled water and make up to 10 ml.

VII. Lactic dehydrogenase, LDH (0.5 mg. protein/ml.):
Dilute the stock solution with 2.1 M ammonium sulphate solution.

Stability of the solutions

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

Procedure

Experimental material and deproteinization

Preliminary remarks: DPNH is extracted from the sample with alkali, since it is destroyed by acid conditions. To obtain complete extraction of the DPNH, especially from muscle, the mixture of tissue powder and KOH must be heated. This procedure also ensures complete inactivation of the enzymes. The heating should not last more than a minute because DPNH rapidly decomposes in hot alcohol. The addition of alcohol expedites the extraction of DPNH and the denaturation of the proteins. After extraction with alcoholic alkali the neutralized extracts are less turbid than those obtained after extraction with aqueous alkali.

Method:

Prepare tissue as described on p. 47; pipette 2 ml. alcoholic KOH (solution I) into small tubes, seal tightly with polyethylene stoppers and weigh. Pre-heat in a water bath at 70°C and, while stirring vigorously (heated magnetic stirrer), introduce 200 to 400 mg. of finely powdered tissue as quickly as possible without allowing the tissue particles to adhere to the walls of the tube (any traces of undenatured tissue will decompose DPNH after neutralization of the extract). Immediately stopper the tube tightly, heat for 60 sec. in the 70°C water bath, then cool quickly in an ice bath and reweigh to obtain the exact weight of tissue added. After 10 min. neutralize the extract by slowly adding 0.5 ml. triethanolamine-HCl-phosphate mixture (III) per ml. extract, while stirring and cooling in ice. The pH should be 7.8; it is recommended that the required amount of solution III be previously determined. Allow to stand for 20 min. at room temperature to obtain better flocculation of the denatured protein. Centrifuge at 20000 to 40000 g for 10 min. Use the almost clear supernatant for the measurements.

Deproteinize blood without heating and use the more concentrated alcoholic KOH to avoid excessive dilution. Prepare centrifuge tubes containing 0.5 ml. 1 N alcoholic KOH per ml. of blood and squirt the blood in with vigorous stirring. Allow to stand for 5 min. at room temperature and then cool in an ice bath. To precipitate the haemochromogens immediately mix 0.5 ml. saturated ammonium sulphate solution (V) per ml. into the deep-brown solution, and follow by 0.2 ml. triethanolamine-HCl solution (IV) per ml. to neutralize the mixture.

The pH should be *ca.* 7.8. Centrifuge off the denatured protein (10 min. at 10000 g). Use the nearly clear supernatant undiluted for the measurements.

Mitochondria: Add 0.5 ml. 1 N alcoholic KOH (solution II) to 1 ml. of mitochondrial suspension. Allow to stand for 10 min. at room temperature and then cool in an ice bath. Adjust to pH 7.8 with *ca.* 1.5 ml. triethanolamine-HCl-phosphate-mixture (III). Further operations as described for tissue.

Spectrophotometric measurements

Wavelength: 340 or 334 mμ (not 366 mμ); light path: 2 cm. or 4 cm.; final volume: 2.01 ml.; (with 4 cm. light path: 4.02 ml.; double the volume of all 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 ml. extract
0.005 ml. pyruvate solution (VI).

Mix and read the optical density E_1. Mix in

0.005 ml. LDH suspension (VII)

and after about 3 min. read the final optical density E_2. $\Delta E = E_1 - E_2$ is used for the calculations.

Calculations

The calculations are as for DPN, see p. 530.

Sources of Error and Specificity

Muscle lactic dehydrogenase reacts not only with DPNH, but also to a slight extent with TPNH. The rate of the reaction with TPNH decreases rapidly with increasing pH, so that at pH 7.8 lactic dehydrogenase reacts 2000 times faster with DPNH than with TPNH[3]. By use of small amounts of LDH it is possible to determine DPNH quantitatively at this pH without interference from additional oxidation of TPNH.

A source of error has recently been described by *Lowry et al.*[4]: DPNH is slowly oxidized by the haemochromogen formed from haemoglobin, which is soluble in alkaline extracts. This reaction has also been demonstrated experimentally by us with alcoholic KOH extracts. On the other hand, DPNH is stable in neutral extracts or those in which most of the haemochromogen has been removed by ammonium sulphate precipitation. This source of error is reduced to a minimum in the method described here, since the extract is only alkaline for a relatively short time.

Several methods are given in the literature for the extraction of reduced pyridine nucleotide. For example, extraction with aqueous KOH at 100° C[5], with buffer (pH 8.6) at 100° C[6,7], or with Na_2CO_3 solution (pH 10)[8]. The differences in the published values for the DPNH content of tissues may be due to imperfections in the methods used.

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[3] *F. Navazio, B. B. Ernster* and *L. Ernster*, Biochim. biophysica Acta *26*, 416 [1957].
[4] *V. H. Lowry, J. V. Passoneau* and *M. K. Rock*, J. biol. Chemistry *236*, 2756 [1961].
[5] *H. Holzer, S. Goldschmidt, W. Lamprecht* and *E. Helmreich*, Hoppe-Seylers Z. physiol. Chem. *297*, 1 [1954].
[6] *M. A. Spirte* and *H. J. Eichel*, Arch. Biochem. Biophysics *53*, 308 [1954].
[7] *L. A. Jedeikin* and *S. Weinhouse*, J. biol. Chemistry *213*, 271 [1958].
[8] *K. B. Jakobson* and *L. Astradan*, Arch. Biochem. Biophysics *71*, 69 [1957].

Other Methods for the Determination of DPNH

Another, frequently used method for the determination of DPNH is the oxidation of DPNH with acetaldehyde, which is catalysed by alcohol dehydrogenase (ADH):

(2) $$\text{DPNH} + \text{H}^+ + \text{acetaldehyde} \rightleftharpoons \text{DPN}^+ + \text{ethanol}$$

However, alcohol dehydrogenase preparations may contain small, but variable amounts of a TPN-specific alcohol dehydrogenase, resulting in the oxidation of TPNH at high ADH concentrations. Glycerol-1-phosphate dehydrogenase reacts specifically with DPNH[9]:

(3) $$\text{DPNH} + \text{H}^+ + \text{dihydroxyacetone phosphate} \rightleftharpoons \text{DPN}^+ + \text{glycerol-1-phosphate}$$

In this case, the equilibrium also lies far on the side of DPN.

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9) *H. Holzer, D. Busch* and *H. Kröger*, Hoppe-Seylers Z. physiol. Chem. *313*, 184 [1958].