Diphosphopyridine Nucleotide (DPN)

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Principle

DPN is reduced by ethanol and alcohol dehydrogenase to DPNH:

(1) $$\text{Ethanol} + \text{DPN}^+ \rightleftharpoons \text{acetaldehyde} + \text{DPNH} + \text{H}^+$$

The equilibrium constant

$$K_{pH} = \frac{K}{[H^+]} = \frac{[\text{acetaldehyde}] \times [\text{DPNH}]}{[\text{ethanol}] \times [\text{DPN}^+]}$$

is $K_7 = 10^{-4}$ at pH 7, $K_{8.8} = 10^{-2}$ at pH 8.8 (25°C). Therefore the equilibrium is in favour of the left side of equation (1).

Quantitative reduction of DPN can be obtained: 1. by an alkaline assay medium, 2. by a high ethanol concentration and 3. by trapping the acetaldehyde formed with semicarbazide. An almost complete reduction of small amounts of DPN occurs without semicarbazide at alkaline pH and high ethanol concentration. It can be calculated from the equilibrium constant that at pH 8.8 and with 0.5 M ethanol, 10^{-4} mole DPN will be 97% and 10^{-5} mole DPN 99.7% reduced, assuming that the sample contains no acetaldehyde or DPNH initially. DPNH is destroyed on extraction with perchloric acid by the acid conditions. The required ethanol concentration is lower in the presence of semicarbazide. A pyrophosphate buffer is used because pyrophosphate binds heavy metal ions, which may inhibit the alcohol dehydrogenase.

Reagents

1. Perchloric acid, A. R. sp. gr. 1.67; *ca.* 70% (w/w)
2. Potassium hydroxide, A. R., 3 N
3. Dipotassium hydrogen phosphate, K_2HPO_4, A. R.
4. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$, A. R.
5. Ethanol, absolute, A. R.
6. Alcohol dehydrogenase, ADH
   crystalline, from yeast[1], suspension in 2.4 M ammonium sulphate solution containing 3% $Na_4P_2O_7$ and 1% glycine, pH *ca.* 8. Commercial preparation, see p. 969.

   Purity of the enzyme preparation

   The specific activity should be about 10000 units/mg. according to *Bücher*[2] or *ca.* 180 units/mg. according to *Racker*[1]. For the definition of units, see pp. 32 and 33. The preparation must be free from TPN-specific dehydrogenases.

Preparation of Solutions

I. Perchloric acid (0.6 N):
   Dilute 5.2 ml. $HClO_4$ (sp. gr. 1.67) to 100 ml. with doubly distilled water.

II. Perchloric acid (3 N):
   Dilute 26 ml. $HClO_4$ (sp. gr. 1.67) to 100 ml. with doubly distilled water.

III. Dipotassium hydrogen phosphate (1 M):
   Dissolve 17.4 g. K_2HPO_4 in doubly distilled water and make up to 100 ml.

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[1] *E. Racker*, J. biol. Chemistry *184*, 313 [1950].

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

IV. Pyrophosphate buffer (0.1 M; pH 8.8):
Dissolve 4.5 g. $Na_4P_2O_7 \cdot 10H_2O$ and 0.5 g. semicarbazide hydrochloride in doubly distilled water and make up to 100 ml.

V. Alcohol dehydrogenase, ADH (1.2 mg. protein/ml.):
Dilute the stock suspension with 2.4 M ammonium sulphate solution.

Stability of the solutions

The ADH suspension (V) should be freshly prepared for each series of measurements from a concentrated stock suspension. It is not stable for more than a few hours. All the other solutions keep for several months if protected from bacterial contamination.

Procedure

Experimental material and deproteinization

The pyridine nucleotides DPN and TPN are extracted from animal tissue, blood or mitochondria by perchloric acid. The final perchloric acid concentration should be about 0.5 M. Therefore 5 ml. 0.6 N $HClO_4$ are added to 1 g. tissue or 1 ml. blood.

Obtain *tissue* by the "quick-freeze" method (refer to p. 47) and while still in frozen state powder finely in a mortar. Prepare a centrifuge tube or small glass beaker with sufficient 0.6 N perchloric acid solution (I) to give a ratio of weight of tissue to $HClO_4$ of 1 : 5. Weigh, introduce the frozen tissue powder with vigorous stirring (magnetic stirrer) and reweigh. Adjust the ratio of tissue to $HClO_4$ to about 1 : 5 by addition of more tissue or perchloric acid.

Squirt *blood* directly from the syringe into the perchloric acid solution (I).

Deproteinize suspensions of *mitochondria* with 3 N perchloric acid solution (II) to avoid too great a dilution: Add 0.2 ml. 3 M perchloric acid solution (II) for every 1 ml. of the mitochondrial suspension.

Centrifuge for 5 min. at 3000 to 5000 g to remove the protein. Suck off about 2 ml. of the supernatant with a pipette, taking care not to include protein, and add to a 10 ml. flask cooled in ice. Pipette in 0.2 ml. K_2HPO_4 solution (III) and, while stirring vigorously (magnetic stirrer), allow 3 N KOH to run in from a fine capillary pipette until the pH reaches 7.2 to 7.4. Allow the $KClO_4$ to sediment and then pipette off samples from the supernatant for the measurements.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 2.04 ml.; room temperature. Measure against air or water.

Pipette successively into the cuvette:

1.00 ml. extract
1.00 ml. pyrophosphate buffer (solution IV)
0.02 ml. ethanol.

Mix, read optical density E_1. Start the reaction by mixing in

0.02 ml. ADH suspension (V)

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

Calculations

$$\frac{\Delta E \times 2.04}{d \times \varepsilon}\left(1 + \frac{V_2}{V_1}\right)\left(1 + \frac{V_4 + V_5}{V_3}\right) = \mu\text{moles DPN/ml. sample}$$

where

d = light path of the cuvette [cm.]
ε = extinction coefficient for DPNH [cm.²/μmole]; 6.22 at 340 mμ; 3.30 at 366 mμ
V_1 = volume of the sample = weight/density [ml.]
V_2 = volume of $HClO_4$ required for deproteinization [ml.]
V_3 = volume of deproteinized supernatant taken [ml.]
V_4 = volume K_2HPO_4 solution added [ml.]
V_5 = volume of KOH required for neutralization [ml.]
2.04 = volume of the assay mixture [ml.]

The amount of DPN in the cuvette is $\frac{\Delta E \times 2.04}{d \times \varepsilon}$. This is multiplied by the dilution factors so that amount of DPN per unit volume of the sample is obtained.

As the density of tissue and blood is nearly 1, the weight (in g.) and the volume (in ml.) can be virtually interchanged. Therefore DPN/ml. ≈ DPN/g.

Sources of Error and Specificity

Alcohol dehydrogenase preparations from yeast usually contain small amounts of a TPN-specific alcohol dehydrogenase[3,4]. Interference due to this contamination can be avoided if only small amounts of alcohol dehydrogenase are used. It is therefore possible in an extract containing DPN and TPN to specifically estimate DPN.

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