Ethanol

Determination with Alcohol Dehydrogenase and DPN

Roger Bonnichsen

Principle

Alcohol dehydrogenase (ADH) catalyses the reaction:

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

Reduced diphosphopyridine nucleotide (DPNH) has an absorption maximum at 340 mμ, while diphosphopyridine nucleotide (DPN) has virtually no absorption at this wavelength. In principle therefore, all four reactants can be determined[1,2]. Several workers have developed methods for the determination of ethanol with alcohol dehydrogenase[3-7].

The equilibrium lies to the left at pH 7[8], with a constant K of 1.1×10^{-11} [mole/l.]. The equilibrium is virtually completely displaced towards the right at alkaline pH provided that acetaldehyde is trapped with semicarbazide. The amount of alcohol present can be determined by measurement of the DPNH formed.

Reagents[+]

1. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$, A. R.
2. Semicarbazide hydrochloride, A. R.*)
3. Glycine
4. Sodium hydroxide, 2 N, A. R.
5. Perchloric acid, A. R.; sp. gr. 1.67; ca. 70% (w/w)
6. Diphosphopyridine nucleotide, DPN**)
   free acid; commercial preparation, see p. 1010.
7. Ethanol standard***)
8. Alcohol dehydrogenase, ADH
   commercial preparation, see p. 969.

Preparation of Solutions

I. Buffer solution:

Dissolve 200 g. $Na_4P_2O_7 \cdot 10H_2O$, 50 g. semicarbazide hydrochloride and 10 g. glycine in doubly distilled water, add 200 ml. 2 N NaOH and dilute to 6 litres. Adjust pH to *ca.* 8.8. The solution is stable for a week at room temperature, but absorption of atmospheric CO_2 must be prevented.

[+] Complete reagent kits are available commercially, see p. 1035.

*) The Analar reagent (Hopkins and Williams, Ltd., Chadwell Heath, Essex, England) is free from ethanol.

**) Boehringer, pure

***) From E. Merck, Darmstadt, Germany.

1) *H. Theorell* and *R. Bonnichsen*, Acta chem. scand. *5*, 1105 [1951].

2) *H. Theorell* and *B. Chance*, Acta chem. scand. *5*, 1127 [1951].

3) *T. Bücher* and *H. Redetzki*, Klin. Wschr. *29*, 615 [1951].

4) *R. Bonnichsen* and *H. Theorell*, Scand. J. Clin. Lab. Invest. *3*, 58 [1951].

5) *R. Bonnichsen* and *G. Lundgren*, Acta Pharmacol. Toxicol. *13*, 256 [1957].

6) *N. G. Brink*, *R. Bonnichsen* and *H. Theorell*, Acta Pharmacol. Toxicol. *10*, 223 [1954].

7) *F. Lundquist* in *D. Glick:* Methods of Biochemical Analysis. Interscience, New York, London 1959, Vol. 7, p. 217.

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

II. Perchloric acid (3.4% w/v):
Dilute 29 ml. 70% $HClO_4$ to 1000 ml. with doubly distilled water.

III. Diphosphopyridine nucleotide (*ca.* 0.15 M β-DPN):
Dissolve 120 mg. DPN in 1 ml. doubly distilled water. The solution is stable for a week in a refrigerator.

IV. Ethanol standard:
Ethanol solutions (0.8—1.9 mg./ml.) in ampoules are commercially available*). Opened ampoules must be used on the same day. The standard solutions can also be prepared in the laboratory with proper precautions.

V. Alcohol dehydrogenase, ADH (*ca.* 30 mg. protein/ml.):
Commercially available**), crystalline alcohol dehydrogenase is dispatched as a suspension in ammonium sulphate solution. Use the suspension undiluted. It is stable for several months at −20° C. The enzyme can also be prepared in the laboratory[8]. The enzyme activity is measured according to *Dotzauer* et al.[9]. The Boehringer enzyme preparation contains 2000 to 2500 units/mg.

Procedure

Deproteinization

Add blood samples (80—120 mg.) to centrifuge tubes containing 1 ml. perchloric acid solution (II). The blood is either weighed in a tared capillary (*e.g.* according to *Widmark*) or pipetted directly. After mixing with the perchloric acid allow all the samples to stand overnight or at least 1 to 2 hours at room temperature. Centrifuge and use the clear supernatants for the analysis.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 3.06 ml. Measure against air or water. 40 samples can be measured in a series. Each series also includes 6 blood samples containing no alcohol (blood blanks).

Pipette into test tubes, or better still into cuvettes:

Tube No.	1 2 Blank samples	3 4 5 6 Standards	7—47 Samples
Buffer (soln. I)	3.00 ml.	3.00 ml.	3.00 ml.
DPN soln. (III)	0.01 ml.	0.01 ml.	0.01 ml.
Blood blank, deproteinized	0.04 ml.	0.04 ml.	—
Ethanol standard (soln. IV)	—	0.005 ml.***)	—
Sample, deproteinized	—	—	0.04 ml.

Mix with a plastic rod and read the optical density E_1. Mix into each tube

0.01 ml. ADH suspension (V) (*ca.* 200—300 μg. protein****),

allow to stand for 70 (at the most 90) min. at 22—26° C and then read the optical density E_2. Use the difference $\Delta E = E_2 - E_1$ for the calculations.

*) From E. Merck, Darmstadt, Germany
**) From C. F. Boehringer und Soehne, Mannheim, Germany.
***) From different ampoules of the Merck standard solutions, *e.g.* a) 0.8 mg. alcohol/ml. (= 4 μg.); b) 1.2 mg./ml. (6 μg.); 1.5 mg./ml. (7.5 μg.); d) 1.8 mg./ml. (9 μg.).
****) The absorption of the enzyme (Boehringer) at 340 mμ can be neglected.

9) *G. Dotzauer, H. Redetzki, K. Johannsmeier* and *T. Bücher*, Dtsch. Z. gerichtl. Med. *41*, 15 [1952].

Calculations •

Subtract the mean of the ΔE values for the blank samples from the ΔE values for the samples and the standards. Plot the corrected ΔE values for the standards (ordinate) against the μg. of ethanol added (abscissa). Under the conditions described here the corrected ΔE values are proportional to an ethanol content of up to 0.4% in the original blood sample. If the temperature (22–26° C) is adhered to there is little change in the slope of the standard curve between each series of estimations.

The ethanol content of the unknown samples are obtained by reading off from the standard curve the μg. of ethanol corresponding to the corrected ΔE values and multiplying by the dilution factor (see also p. 37).

Measurements in Serum and Urine

Serum (10 μl.) or urine (5 μl.) need not be deproteinized and can be pipetted directly into the buffer solution. For serum analysis the 6 blank solutions should contain 10 μl. alcohol-free serum. Urine occasionally absorbs at 340 mμ. The absorption due to the samples is corrected for by measurement of the optical density before and after the enzymatic reaction (E_1 and E_2).

Sources of Error and Specificity

The increase in optical density at 340 mμ after addition of ADH must not exceed 0.02–0.03 in the blank samples. If this is not the case, then alcohol is present as a contaminant in the reagents. If the standard curve is not a straight line it is usually due to too low a DPN concentration.

Apart from ethanol, only the primary and secondary aliphatic alcohols, especially n-propanol and n-butanol, react under the conditions described here. Concentrations as low as 1 mg. ethanol/1000 ml. can be estimated. A greater sensitivity can be obtained if instead of the absorption at 340 mμ the fluorescence of DPNH is measured[10].

Determination with Alcohol Dehydrogenase and the 3-Acetylpyridine Analogue of DPN (AP-DPN)

Helmut Holzer and Hans-Dieter Söling

The spectrophotometric determination of ethanol with yeast alcohol dehydrogenase (ADH) and AP-DPN, without the use of a trapping agent for acetaldehyde, is possible because of the more favourable position of the redox potential of the system AP-DPN$^+$/ AP-DPNH in contrast to that of DPN$^+$/DPNH (refer to p. 289)

Principle

Alcohol dehydrogenase catalyses the reaction:

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

At pH 7 the equilibrium of the reaction lies to the left. At pH 9 the equilibrium constant is about 1 without taking into account the H^+ ions. Alkaline pH therefore favours the oxidation of ethanol. As the affinity of ADH for ethanol is low ($K_M = 5.5 \times 10^{-2}$ M), high concentrations of ADH and AP-DPN must be used in order to obtain a quantitative oxidation.

[10] *H. Theorell, A. P. Nygård* and *R. Bonnichsen*, Acta chem. scand. *9*, 1148 [1955]; *H. Theorell* Scand. J. Clin. Lab. Invest. *10*, Suppl. 31 [1957].

Reagents

1. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10\ H_2O$, A. R.
2. Glycine, A. R.
3. Hydrochloric acid, A. R., 2 N
4. Ethylene-diamine-tetra-acetic acid, EDTA
   sodium salt, EDTA-$Na_2H_2 \cdot 2\ H_2O$ (*e.g.* Titriplex III from E. Merck, Darmstadt, Germany).
5. 3-Acetylpyridine analogue of diphosphopyridine nucleotide, AP-DPN *)
6. Alcohol dehydrogenase, ADH
   from yeast, crystalline, suspended in 2.4 M ammonium sulphate solution containing 3% sodium pyrophosphate and 1% glycine; commercial preparation, see p. 969.

Preparation of Solutions

I. Sodium pyrophosphate buffer (0.075 M; pH 8.9):
Dissolve 3.3 g. sodium pyrophosphate $\cdot 10\ H_2O$, 186 mg. EDTA-$Na_2H_2 \cdot 2\ H_2O$ and 0.15 g. glycine in 50 ml. doubly distilled water, adjust to pH 8.9 with *ca.* 0.5 ml. 2 N HCl (glass electrode) and dilute to 100 ml. with doubly distilled water.

II. 3-Acetylpyridine analogue of diphosphopyridine nucleotide, (*ca.* 1.5×10^{-2} M AP-DPN):
Dissolve 10 mg. AP-DPN in 1 ml. doubly distilled water.

III. Alcohol dehydrogenase, ADH (30 mg. protein/ml.):
Use the commercially available suspension in 2.4 M ammonium sulphate solution containing 3% sodium pyrophosphate and 1% glycine.

Procedure

For treatment of the samples (*e.g.* blood), see p. 286.

Spectrophotometric measurements

Wavelength: 366 mμ; glass cuvettes, light path: 0.5 cm.; final volume: 0.45 ml. The absorption maximum of AP-DPNH is at 363 mμ [1]. The light path and final volume may be altered so as to make the assay more sensitive. Measure against distilled water. Place the cuvettes in either a constant temperature cuvette holder or a water bath at 37°C.

Warm the buffer and sample to 37°C before the assay.

Pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
0.27 ml. buffer (solution I)	0.27 ml. buffer (solution I)
0.10 ml. AP-DPN solution (II)	0.10 ml. AP-DPN solution (II)
sample + water to 0.42 ml.	water to 0.42 ml.

Observe the optical densities of both cuvettes. If the optical density change in both cuvettes is not more than 0.001 to 0.002 per 30 sec., mix into both cuvettes

0.03 ml. ADH suspension (III) (*ca.* 0.9 mg. protein).

After 40 to 60 min. the optical density is usually constant or the experimental cuvette shows the same very small increase in optical density with time as the control cuvette. A cuvette, which contains all the constituents of the assay mixture except for the enzyme,

*) Obtainable from the Pabst Laboratories, Milwaukee, Wisconsin, USA.

usually shows no significant change in optical density with time. The optical density difference between the experimental and control cuvette on completion of the reaction minus the optical density difference between the experimental and control cuvette before the start of the reaction with ADH gives the ΔE required for the calculations.

Calculations

$$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles ethanol in the cuvette}$$

ΔE is the increase of optical density on addition of ADH, corrected as described above. The extinction coefficient ε of AP-DPNH is 9.1 cm.^{2}/μmole[1] at 366 mμ, d is the light path of the cuvette in cm. and V is the final volume of the assay mixture in ml.

Appendix

Initial velocities of the oxidation of ethanol with AP-DPN and DPN

With 1×10^{-4} moles/l. AP-DPN or DPN in 0.068 M pyrophosphate buffer pH 9.0 and with 0.75 mg. protein/l. the ratio of the initial velocities of ethanol oxidation $V_{DPN}:V_{AP-DPN}$ is 10.4:1.

Michaelis constants (K_M)

The reaction rates with 2.6×10^{-3} to 3.4×10^{-2} M ethanol or with $1.25 - 10^{-5}$ to 5×10^{-4} M AP-DPN were measured in 0.075 M pyrophosphate-glycine buffer (pH 9.0) at 23°C. The constants were obtained according to *Lineweaver* and *Burk*[2]. With 4.3×10^{-3} M ethanol the K_M for AP-DPN is 1.67×10^{-3} M; with 2.5×10^{-4} M AP-DPN the K_M for ethanol is 5.5×10^{-2} M.

Equilibrium constant[3]

$$\text{The equilibrium constant } K = \frac{[\text{AP-DPNH}] \times [\text{acetaldehyde}] \times [H^+]}{[\text{AP-DPN}^+] \times [\text{ethanol}]}$$

was calculated to be 1.1×10^{-9} M at 25°C (mean of four measurements). Ethanol was determined by means of the assay described above. AP-DPN was determined by complete reduction to AP-DPNH with malic dehydrogenase at pH 9.5 and excess malate (refer to p. 332). The H^+ ion concentration was determined with a glass electrode after the equilibrium was reached. The equilibrium concentrations of AP-DPN and acetaldehyde were obtained from the spectrophotometrically measured concentration of AP-DPNH at equilibrium.

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[1] *J. M. Siegel, G. A. Montgomery* and *R. M. Bock*, Arch. Biochem. Biophysics *82*, 288 [1959].
[2] *H. Lineweaver* and *D. Burk*, J. Amer. chem. Soc. *56*, 658 [1934].
[3] *H. Holzer* and *H. D. Söling*, Biochem. Z. *336*, 201 [1962].