L-Aspartic Acid and L-Asparagine

Determination with Glutamate-Oxaloacetate Transaminase and Malic Dehydrogenase

Gerhard Pfleiderer

Principle

Glutamate-oxaloacetate transaminase (GOT), in the presence of L-aspartic acid, converts α-oxoglutaric acid to L-glutamic acid.

(1) α-Oxoglutarate + L-aspartate $\overset{\text{GOT}}{\rightleftharpoons}$ L-glutamate + oxaloacetate

Malic dehydrogenase (MDH) reduces oxaloacetate to L-malate in the presence of reduced diphosphopyridine nucleotide (DPNH):

(2) Oxaloacetate + DPNH + H^+ $\overset{\text{MDH}}{\rightleftharpoons}$ L-malate + DPN^+

The decrease in the optical density of DPNH can be followed spectrophotometrically at 340 or 366 mμ. The equilibrium of reaction (2) ($K = 4.3 \times 10^4$ l./mole at pH 7.2 and 22°C) lies greatly in favour of malate formation[1]. The Michaelis constant of the enzyme with respect to L-aspartate is very small. L-Aspartate is quantitatively converted to L-(+)-malate with the oxidation of a stoichiometric amount of DPNH[2].

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$, A. R.
3. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see ρ. 1011.
4. α-Oxoglutaric acid
   commercial preparation, see p. 1024.
5. Sodium hydroxide, A. R., 2 N
6. Malic dehydrogenase, MDH
   from pig heart[3,4], suspension in 2.8 M ammonium sulphate solution; commercial preparation, see p. 988.
7. Glutamate-oxaloacetate-transaminase, GOT
   from pig heart[5], suspension in 1.6 M ammonium sulphate solution; commercial preparation, see p. 976.

Purity of the enzyme preparations

MDH and GOT must be as free as possible from glutamic dehydrogenase activity. Otherwise, as both enzymes are usually stored as ammonium sulphate suspensions, with excess α-oxoglutarate and DPNH, a slow reductive amination to give glutamic acid and a corresponding oxidation of DPNH would occur. However, both enzymes are easily prepared in a virtually pure state[3–5] and can be stored for several weeks at 0–4°C without loss of activity.

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[1] *J. R. Stern, S. Ochoa* and *F. Lynen*, J. biol. Chemistry *198*, 313 [1952].
[2] *G. Pfleiderer, W. Gruber* and *Th. Wieland*, Biochem. Z. *326*, 446 [1955].
[3] *R. G. Wolfe* and *J. B. Neilands*, J. biol. Chemistry *221*, 61 [1956].
[4] *G. Pfleiderer* and *E. Hohnholz*, Biochem. Z. *331*, 245 [1959].
[5] *W. T. Jenkins, D. A. Yphantis* and *J. W. Sizer*, J. biol. Chemistry *234*, 51 [1959].

Preparation of Solutions

I. Phosphate buffer (M/15; pH 7.2):
a) Dissolve 11.876 g. $Na_2HPO_4 \cdot 2H_2O$ in doubly distilled water and make up to 1000 ml.
b) Dissolve 9.078 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml.
Mix a) and b) in the ratio of 72 : 28 volumes.

II. Reduced diphosphopyridine nucleotide (*ca.* 1.2×10^{-2} M β-DPNH):
Dissolve 50 mg. DPNH-Na_2 in 5 ml. doubly distilled water.

III. α-Oxoglutarate (0.1 M):
Dissolve 1.46 g. α-oxoglutaric acid in *ca.* 50 ml. doubly distilled water with gentle warming, neutralize with 2 N NaOH and dilute with doubly distilled water to 100 ml.

IV. Malic dehydrogenase, MDH (2.5 mg. protein/ml.):
If necessary, dilute the stock suspension with 2.8 M ammonium sulphate solution.

V. Glutamate-oxaloacetate transaminase, GOT (2.5 mg. protein/ml.):
If necessary, dilute the stock suspension with 1.6 M ammonium sulphate solution.

Procedure

Preliminary treatment of the experimental material

Treat tissue extracts and protein hydrolysates as described on p. 379.

To determine asparagine in the presence of aspartic acid, heat a portion of the solution for 2 hrs. in a boiling water bath with 1 N HCl, cool, neutralize with 2 N NaOH and dilute with distilled water to a known volume. Use this solution for the assay. The sum of asparagine and aspartic acid is obtained *).

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3 ml.; room temperature. Read against a water blank.

Pipette successively into the cuvette:

2.76 ml. buffer (solution I)
0.10 ml. treated sample
0.05 ml. DPNH solution (II)
0.02 ml. MDH suspension (IV)
0.02 ml. GOT suspension (V).

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

0.05 ml. α-oxoglutarate solution (III).

The reaction is complete in less than 10 min. Read optical density E_2. If the optical density should continue to slowly decrease, obtain E_2 by extrapolation from start of the reaction according to p. 39.

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*) For an enzymatic determination of asparagine the sample is not hydrolysed. Instead asparaginase is added to the test mixture after the reaction with aspartate is finished. The further decrease in optical density corresponds to the asparagine content of the sample. *D. H. Williamson*, unpublished.

Calculations

$E_2 - E_1 = \Delta E$ is used for the calculations. The error due to the dilution of the assay mixture by addition of 0.05 ml. α-oxoglutarate solution is significant with small ΔE values. It is eliminated by multiplying E_1 by $3.00/3.05 \approx 0.98$.

For measurements at 366 mμ

$$\frac{\Delta E \times 3 \times 133}{3.3 \times 10^3} = \text{mg. aspartic acid in the cuvette}$$

where

3.3×10^3 = extinction coefficient of DPNH at 366 mμ [cm.²/mmole]
133 = molecular weight of aspartic acid
3 = test volume [ml.]

For measurement at 340 mμ the extinction coefficient of DPNH is 6.22×10^3 cm.²/mmole.

The asparagine content of the sample is calculated from the difference in the values of samples before and after preliminary acid hydrolysis or from the decrease in optical density after addition of asparaginase, see footnote on p. 382. The molecular weight of asparagine (132.12) and aspartic acid (133.10) are virtually the same.

Specificity and Sources of Error

The transamination reaction is very specific. Only L-aspartate and the sulpho-analogue, cysteic acid, react; the latter much more slowly. The reaction with cysteic acid is linear for a long period of time and therefore can be corrected for by extrapolation according to p. 39. Cysteic acid can arise by the hydrolysis of proteins and peptides containing cysteine and cystine in the presence of oxygen. Concentrations of oxaloacetate higher than 10^{-4} M will interfere because of the additional oxidation of DPNH. As a rule such interference does not occur with tissues because the oxaloacetate concentration is usually low. If necessary, the values for oxaloacetate may be determined separately *) (p. 335) and be subtracted from the value found for aspartate. Alternatively, if the addition of the α-oxoglutarate solution is not made until a steady optical density is obtained then no correction is necessary.

D-Aspartate can be determined if the value for L-aspartate obtained enzymatically is subtracted from the value for total aspartate determined with ninhydrin (after chromatographic or electrophoretic separation of the aspartate).

When applied to protein hydrolysates the values obtained with the method described above were in good agreement with those found by chemical methods after ion-exchange chromatography [2].

*) For the determination of oxaloacetate the optical density is read before and after addition of MDH. The above formula can be used for the calculations, substituting 2.7 instead of 3 and 132 instead of 133.