Glyoxylate

Helmut Klotzsch and Hans-Ulrich Bergmeyer

A simple and specific determination of glyoxylic acid is impossible by purely chemical means. As glyoxylic acid is of interest as a possible metabolite involved in the respiration of green plants, its rapid and largely specific enzymatic estimation represents an important advance.

Principle

Glyoxylic acid is reduced by DPNH and glyoxylic acid reductase to glycollic acid and DPN[1,2]:

(1) $CHO-COOH + DPNH + H^+ \rightleftharpoons CH_2OH-COOH + DPN^+$

The decrease of optical density at 366 or 340 mμ due to the oxidation of DPNH is a measure of the reaction. The equilibrium lies far to the right and the reaction proceeds stoichiometrically.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Dipotassium hydrogen phosphate, K_2HPO_4, A. R., anhydrous
3. Sodium hydrogen carbonate, $NaHCO_3$, A. R., anhydrous
4. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
5. Glyoxylic acid reductase, Gly-R
   crystalline, from spinach leaves; suspension in 1.5 M ammonium sulphate solution; specific activity at least 50 units*)/mg.; commercial preparation, see p. 982.
6. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)
7. Potassium carbonate, K_2CO_3, A. R., anhydrous

Purity of the enzyme preparation

Glyoxylic acid reductase must not contain more than 0.01% glycollic acid oxidase, lactic dehydrogenase, DPNH oxidase and alcohol dehydrogenase (relative to its own specific activity).

Preparation of Solutions

I. Phosphate buffer (0.1 M; pH 6.4):
   a) Dissolve 1.36 g. KH_2PO_4 in doubly distilled water and make up to 100 ml.
   b) Dissolve 1.74 g. K_2HPO_4 in doubly distilled water and make up to 100 ml.
   Mix 50 ml. solution a) with 21 ml. solution b). Check the pH (glass electrode).

II. Sodium hydrogen carbonate (5% w/v):
   Dissolve 5 g. $NaHCO_3$ in doubly distilled water and make up to 100 ml.

III. Reduced diphosphopyridine nucleotide (*ca.* 0.012 M β-DPNH):
   Dissolve 10 mg. DPNH in 1 ml. $NaHCO_3$ solution (II).

IV. Glyoxylic acid reductase, Gly-R (1 mg. protein/ml.):
   Dilute the stock suspension with 1.5 M ammonium sulphate solution.

*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min. at 25°C.

1) *I. Zelitch*, J. biol. Chemistry *216*, 553 [1955].
2) *H. Holzer* and *A. Holldorf*, Biochem. Z. *329*, 292 [1957].

V. Perchloric acid (*ca.* 6% w/v):
Dilute 5.2 ml. 70% $HClO_4$ to 100 ml. with doubly distilled water.

VI. Potassium carbonate (1.0 M):
Dissolve 13.8 g. anhydrous K_2CO_3 in doubly distilled water and make up to 100 ml.

Stability of the solutions

Store all solutions and suspensions, stoppered, in a refrigerator at 0 to 4°C. They keep for severa weeks in this state. Prepare the DPNH solution freshly each week.

Procedure

Deproteinization

Material containing protein, such as plant extracts, must be deproteinized before the analysis. Pipette successively into a centrifuge tube:

5 ml. ice-cold perchloric acid solution (V)
5 ml. extract.

Mix thoroughly with a thin glass rod and centrifuge for 10 min. at 3000 g. Suspend the precipitate in 3 ml. doubly distilled water, centrifuge again and combine the supernatants. Free the supernatant from excess perchloric acid, for example, adjust

9 ml. supernatant

to between pH 6.5 and 7 with

potassium carbonate solution (VI).

Allow to stand for 15 min. in an ice bath and filter off the precipitate of $KClO_4$. After warming to *ca.* 25°C, use 0.1 ml. of this solution for the assay.

Spectrophotometric measurements

Wavelength: 366 mμ or 340 mμ; light path: 1 cm.; final volume: 3.0 ml.; room temperature. Measure against the control.
Pipette successively into the cuvettes:

Experimental	*Control*
1.00 ml. phosphate buffer (solution I)	1.00 ml. phosphate buffer (solution I)
1.83 ml. doubly distilled water	1.90 ml. doubly distilled water
0.05 ml. DPNH solution (III)	—
0.10 ml. deproteinized sample	0.10 ml. deproteinized sample.

Mix thoroughly with a plastic rod flattened at one end and read the optical density E_1. Mix into the experimental cuvette

0.02 ml. Gly-R suspension (IV)

and follow the decrease in optical density. At the end of the reaction (20 to 35 min.) read the optical density E_2. $E_1 - E_2 = \Delta E$ is used for the calculations.

Calculations

With a final volume in the cuvette of 3.0 ml. (refer to p. 37)

$$\text{at 340 m}\mu\text{:}\quad \frac{\Delta E \times 3.0}{6.22} = \mu\text{moles glyoxylate/assay mixture}$$

$$\text{at 366 m}\mu\text{:}\quad \frac{\Delta E \times 3.0}{3.3} = \mu\text{moles glyoxylate/assay mixture}$$

To obtain the glyoxylic acid concentration per ml. of sample, the dilutions occurring on deproteinization and neutralization must be allowed for. To convert from μmoles to μg. multiply by the molecular weight of glyoxylic acid (74).

Example

An extract (5 ml.) was deproteinized with 5 ml. perchloric acid. After combining the supernatant and the washings, the volume was 9.0 ml. For neutralization 1.2 ml. of potassium carbonate solution was required. Therefore 10.2 ml. filtrate corresponds to 5 ml. original extract. 0.1 ml. of filtrate was taken for the assay.

The following optical densities were measured at 366 mμ: $E_1 = 0.300$; $E_2 = 0.126$; $\Delta E = 0.174$. 1 ml. of the sample therefore contained:

$$\frac{0.174 \times 3 \times 10.2 \times 74}{3.3 \times 0.1 \times 5} = 239 \text{ μg. glyoxylic acid/ml. sample}$$

Specificity and Sources of Error

Glyoxylic acid reductase also reacts with hydroxypyruvate; the reaction product is D-glycerate. Therefore in the glyoxylic acid determination any hydroxypyruvate in the sample reacts quantitatively. If glyoxylate and hydroxypyruvate have to be distinguished, then the D-glycerate formed from the latter is estimated according to *Kattermann*, *Holldorf* and *Holzer* (see p. 220).

The enzyme does not react with pyruvate, α-oxoglutarate, oxaloacetate, mesoxalate, phenylglyoxylate or acetaldehyde. Erroneous results are obtained if the enzyme preparation is not sufficiently pure.