α-Oxoglutarate

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The enzymic determination of α-oxoglutarate with reduced diphosphopyridine nucleotide (DPNH) and glutamic dehydrogenase was developed by *Wallenfels* and *Christian*[1], after the enzyme had been isolated in a pure state and crystallized[2,3]. The method was immediately used for metabolic studies (*e.g.* refer to[4–12]), because in contrast to the methods then available (*e.g.* refer to[13,14]) it has the advantage of being simple and specific.

Principle

Glutamic dehydrogenase (GlDH) catalyses the reaction[15]:

(1) α-Oxoglutarate + DPNH + NH_4^+ ⇌ glutamate + DPN^+ + H_2O

With an excess of NH_4^+ ions and DPNH α-oxoglutarate is quantitatively converted to glutamate. For each mole of α-oxoglutarate 1 mole of DPNH is oxidized. The decrease in the optical density at 340 or 366 mμ due to the oxidation of DPNH is a measure of the reaction.

Reagents*)

1. Tripotassium phosphate, K_3PO_4, anhydrous
2. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
3. Sodium hydrogen carbonate, $NaHCO_3$; 1% (w/v)
4. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
5. Glutamic dehydrogenase, GlDH
   crystalline, from ox liver, suspension in 2.8 M ammonium sulphate solution. Commercial preparation, see p. 978.

Purity of the enzyme preparation

The GlDH preparation should have a specific activity of at least 3 units**)/mg. The maximum allowable contamination with other enzymes is: 0.5% lactic dehydrogenase; 0.15% glycerol-

*) Complete reagent kits are available commercially, see p. 1036.
**) A unit is the amount of enzyme which oxidizes 1 μmole glutamate in 1 min.

1) *K. Wallenfels* and *W. Christian*, unpublished.
2) *H. J. Strecker*, Arch. Biochem. Biophysics *32*, 448 [1951].
3) *J. A. Ohlson* and *C. B. Anfinsen*, J. biol. Chemistry *197*, 67 [1952]; *202*, 841 [1953].
4) *W. Lamprecht*, Ph. D.-Thesis, Technische Hochschule München, 1954.
5) *A. Engelhardt-Gölkel*, *R. Prosiegel*, *W. Seitz* and *K. Stulfauth*, Lecture to the 1st European Congress for Clinical Chemistry, Amsterdam 1954.
6) *W. Seitz*, *A. Engelhardt-Göckel* and *I. Schaffry*, Klin. Wschr. *33*, 228 [1955].
7) *H. Redetzki*, *H. Bloedorn* and *H.-W. Bansi*, Klin. Wschr. *34*, 845 [1956].
8) *G. Strohmeyer*, *G. A. Martini* and *V. Klingmüller*, Klin. Wschr. *35*, 385 [1957].
9) *G. Laudahn*, Klin. Wschr. *37*, 850 [1959].
10) *H. Kyank*, Zbl. Gynäkol. *80*, 585 [1958].
11) *H. A. Hötzl*, Ärztl. Wschr. *13*, 726 [1958].
12) *Th. Hockerts*, Monatsschr. Kinderheilkunde *109*, 101 [1961].
13) *M. F. S. El Hawary* and *R. H. S. Thompson*, Biochem. J. *53*, 340 [1953].
14) *S. Markees* and *K. F. Gey*, Helv. physiol. pharmakol. Acta *11*, 49 [1953].
15) *H. v. Euler*, *E. Adler*, *G. Günther* and *N. Bandhu Das*, Hoppe-Seylers Z. physiol. Chem. *254*, 61 [1938].

1-phosphate dehydrogenase; 0.15% malic dehydrogenase (relative to the specific activity of the GlDH).

Preparation of Solutions (for *ca.* 15 determinations)

I. Phosphate (1 M K_3PO_4):
Dissolve 3.2 g. K_3PO_4 in doubly distilled water and make up to 15 ml.

II. Reduced diphosphopyridine nucleotide (*ca.* 8×10^{-3} M β-DPNH):
Dissolve 7 mg. DPNH-Na_2 in 1 ml. 1% $NaHCO_3$ solution.

III. Perchloric acid (*ca.* 0.60 M):
Dilute 5.2 ml. $HClO_4$ (sp. gr. 1.67) to 100 ml. with doubly distilled water.

IV. Glutamic dehydrogenase, GlDH (2 mg. protein/ml.):
If necessary, dilute the stock suspension with 2.8 M ammonium sulphate solution.

Stability of the solutions

Store all solutions in a refrigerator at 0 – 4° C. Prepare the DPNH solution freshly each week. The enzyme suspension keeps for several months.

Procedure

Experimental material

To obtain reproducible results the blood must be withdrawn without constricting the vein. Liver, heart and skeletal muscle should be homogenized in a Potter-Elvehjem or Ultra-Turrax homogenizer (see p. 50, 51) with perchloric acid (solution III). The tissue must be homogenized within 60 sec. of the death of the animal[16].

Deproteinization (blood)

Pipette into a 10 ml. centrifuge tube:

5 ml. perchloric acid solution (III)
5 ml. blood,

mix with a thin glass rod and centrifuge for 5 – 10 min. at *ca.* 3000 g. To

4 ml. of the supernatant

add *ca.*

0.8 ml. phosphate solution (I)

and mix. The pH should be *ca.* 7.6. Allow to stand for 10 min. in ice. Filter off the precipitated $KClO_4$. Allow the solution to warm to room temperature and then use 3.75 ml. for the assay.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 2 cm.; final volume: 3.85 ml.; room temperature. Measure against air or a cuvette containing water. The ammonium ions necessary for the reaction are provided in the addition of the GlDH, which is a suspension in ammonium sulphate solution.

Pipette successively into the cuvette:

3.75 ml. deproteinized sample
0.05 ml. DPNH solution (II),

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[16] *H. Holzer* and *A. Holldorf*, Biochem. Z. *329*, 292 [1957].

mix with a glass rod flattened at one end and read the optical density E_1. Mix in

0.05 ml. GlDH suspension (IV)

and after about 4 min. read the optical density E_2. If the optical density is not constant, continue reading at 1 min. intervals, plot the results and extrapolate (see p. 39) from the zero time (= GlDH addition). $E_1 - E_2 = \Delta E$ is used for the calculations.

The small increase in optical density on addition of the GlDH, caused by the slight turbidity of the enzyme suspension, must be determined for each enzyme preparation. On completion of the reaction mix in a further

0.05 ml. GlDH suspension (IV)

and read E_3. $E_3 - E_2 = \Delta E_E$ is usually very small and must be subtracted from E_2 before calculating $E_1 - E_2 = \Delta E$.

Calculations

According to the usual formula (see p. 37).

$$\text{for 340 m}\mu\text{: } \frac{\Delta E \times 3.85}{6.22 \times 2} = \Delta E \times 0.309 = \mu\text{moles } \alpha\text{-oxoglutarate/cuvette}$$

$$\text{for 366 m}\mu\text{: } \frac{\Delta E \times 3.85}{3.3 \times 2} = \Delta E \times 0.581 = \mu\text{moles } \alpha\text{-oxoglutarate/cuvette}$$

To obtain the α-oxoglutarate content, for example, of blood, the dilutions occurring on deproteinization and on neutralization of the perchloric acid extract must be taken into account. Blood contains *ca.* 80% of its weight of water and 1 ml. blood weighs *ca.* 1.06 g. With 5 ml. blood (= 5.3 g.) the volume of extract after deproteinization is $\frac{5.3 \times 80}{100} + 5 = 9.24$ ml. Of this 4 ml. are neutralized by 0.8 ml. phosphate solution (I) and 3.75 ml. of the neutral extract are taken for assay.

The total dilution is $\frac{9.24}{5} \times \frac{4.8}{4} \times \frac{1}{3.75} = 0.591 : 1$. Therefore the concentration of α-oxoglutarate/ml. blood is obtained by multiplying by 0.591.

For 340 mμ:

$\Delta E \times 0.309 \times 0.591 = \Delta E \times 0.183 =$ μmoles α-oxoglutarate/ml. blood

$\Delta E \times 0.183 \times 146 = \Delta E \times 26.8 =$ μg. α-oxoglutaric acid/ml. blood

(146 is the molecular weight of α-oxoglutaric acid)

For 366 mμ:

$\Delta E \times 0.581 \times 0.591 = \Delta E \times 0.345 =$ μmoles α-oxoglutarate/ml. blood

$\Delta E \times 0.345 \times 146 = \Delta E \times 50.3 =$ μg. α-oxoglutaric acid/ml. blood.

Example

Normal blood (5 ml.) was deproteinized and 0.8 ml. phosphate solution (I) was required to attain pH 7.55 in the perchloric acid supernatant.

The measured values at 366 mμ were: $E_1 = 0.357$; $E_2 = 0.330$; $E_3 = 0.334$.

$E_3 - E_2 = \Delta E_E = 0.004$; $E_2 - \Delta E_E = 0.326$; $\Delta E = 0.357 - 0.326 = 0.031$

$0.031 \times 50.3 = 1.56$ μg. α-oxoglutaric acid/ml. blood or 0.156 mg.%.

Normal Values

Normal values for human blood are *ca.* 1.55 μg. α-oxoglutaric acid/ml. blood (according to[8] 1.4 μg./ml.; according to[6] 1.7 μg./ml.; according to[10] 1.54 μg./ml.). For values in various diseases, see[9,17] and after exposure to X-rays, see[18].

Specificity and Sources of Error

GlDH also catalyses the reductive amination of α-oxovaleric acid to L-norvaline[19]. The rate of this reaction is about 25% of that with α-oxoglutarate. α-Oxobutyric acid and α-oxo-isovaleric acid react at about 2% of the rate of the α-oxoglutarate reaction. Oxaloacetate does not react[19]. Pyruvate reacts about 0.5% as fast as α-oxoglutarate[20].

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17) *F. Gavosto, F. Buffa* and *S. Chiarle*, Acta Cardiol. *13*, 617 [1958].
18) *H. Schön, F. C. Sitzmann* and *G. Barth*, Strahlentherapie *105*, 585 [1958].
19) *K. H. Bässler* and *C.-H. Hammar*, Biochem. Z. *330*, 446 [1958].
20) *H. F. Fisher* and *L. L. McGregor*, J. biol. Chemistry *236*, 791 [1961].