Glycerol

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The determination of glycerol in biological material, food and industrial products usually requires an extensive purification of the sample under investigation in order to remove interfering contaminants. Even after this time consuming purification the chemical methods of analysis are liable to error, especially when the amounts of glycerol present, as in serum, body fluids and tissues, are very small. The enzymatic method for the determination of glycerol is specific[1]. Therefore purification of the sample is unnecessary.

Principle

Glycerol is phosphorylated by glycerokinase (GK) and adenosine triphosphate (ATP) to give L-(−)-glycerol-1-phosphate[2,3]:

(1) $$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{GK} \text{L-(−)-glycerol-1-phosphate} + \text{ADP}$$

The L-(−)-glycerol-1-phosphate formed is oxidized with α-glycerophosphate dehydrogenase[4] (glycerol-1-phosphate dehydrogenase, GDH) and diphosphopyridine nucleotide (DPN):

(2) $$\text{L-(−)-glycerol-1-phosphate} + \text{DPN}^+ \overset{GDH}{\rightleftharpoons} \text{dihydroxyacetone phosphate} + \text{DPNH} + \text{H}^+$$

The amount of DPNH formed is equivalent to the amount of glycerol present. The equilibrium of the indicator reaction (2), which lies far to the left, is displaced in the required direction by working at pH 9.8 and trapping the dihydroxyacetone phosphate (DAP) with hydrazine.

Reagents

1. Hydrazine hydrate, 24% (w/v)
2. Glycine
3. Magnesium chloride, 1 M
4. Potassium hydroxide, A. R., 10 N
5. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
6. Adenosine triphosphate, ATP
   crystalline disodium salt, ATP-$Na_2H_2 \cdot 3\,H_2O$; commercial preparation, see p. 1006.
7. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
8. α-Glycerophosphate dehydrogenase, GDH
   from rabbit muscle[5]; commercial preparation, see p. 981.
9. Glycerokinase, GK
   from pigeon liver[3], *Oospora lactis*[6] or *Candida mycoderma*[7]. Commercial preparation, see p. 980.

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1) *O. Wieland*, Biochem. Z. *329*, 313 [1957].
2) *C. Bublitz* and *E. P. Kennedy*, J. biol. Chemistry *211*, 951 [1954].
3) *O. Wieland* and *M. Suyter*, Biochem. Z. *329*, 320 [1957].
4) *T. Baranowski*, J. biol. Chemistry *180*, 535 [1949].
5) *G. Beisenherz, H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].
6) *E. M. Kauder*, Ph. D.-Thesis, Universität München 1960.
7) *H. U. Bergmeyer, G. Holz, E. M. Kauder, H. Möllering* and *O. Wieland*, Biochem. Z. *333*, 471 [1961].

Purity of the enzyme preparations

Since large amounts of lactate are often present in biological material, both GDH and GK should be as free as possible from lactic dehydrogenase. Preparations from Boehringer und Soehne, Mannheim (Germany), fulfil this requirement.

Preparation of Solutions

All solutions are prepared with doubly distilled water.

I. Buffer (0.2 M glycine, 1 M hydrazine, 2×10^{-3} M Mg^{2+}; pH 9.8):
Mix 20.8 g. hydrazine hydrate (24% w/v) and 1.5 g. glycine with distilled water. Add 0.2 ml. 1 M $MgCl_2$ and adjust to pH 9.8 with several drops 10 N KOH, then dilute to 100 ml. Store in refrigerator in a brown, stoppered bottle. Stable for 4–6 weeks.

II. Perchloric acid (*ca.* 30% w/v):
Dilute 25.5 ml. $HClO_4$ (sp. gr. 1.67) to 100 ml. with distilled water.

III. Perchloric acid (*ca.* 3% w/v):
Dilute 10 ml. solution II to 100 ml. with distilled water.

IV. Adenosine triphosphate (0.05 M ATP):
Dissolve 60.5 mg. ATP-$Na_2H_2 \cdot 3H_2O$ (purity 98%) in about 1 ml. distilled water, neutralize with dilute NaOH and dilute to 2 ml. Less pure ATP preparations are also suitable.

V. Diphosphopyridine nucleotide (*ca.* 0.02 M β-DPN):
Dissolve 34.6 mg. DPN (85% enzymatically active β-DPN) in 2 ml. distilled water.

VI. α-Glycerophosphate dehydrogenase, GDH (*ca.* 7 mg. protein/ml.):
Suspend the twice recrystallized enzyme[5] obtained from rabbit muscle in 2.0 M ammonium sulphate solution; dilute commercial preparations correspondingly.

VII. Glycerokinase, GK (*ca.* 60 units*/ml.):
Since the assay is started with GK, the most concentrated solutions available should be used: with crystalline GK from *Candida mycoderma*[7], 60 units/ml. correspond to about 1 mg. protein/ml. Glycerol, which is added to stabilize GK during purification, must be removed before use. The enzyme is washed several times with 2 M ammonium sulphate solution, and then re-suspended in this solution. It is recommended that the preparation is examined for the presence of glycerol before use. GK from Boehringer und Soehne, Mannheim (Germany) is free from glycerol.

Stability of the solutions

The usual conditions apply for the storage of enzyme and coenzyme solutions (refer to p. 15). Protein suspensions in ammonium sulphate solution should be stored in a refrigerator. The coenzyme solutions can also be stored frozen (deep-freeze) for a long period.

Procedure

Experimental material

Blood is collected by venepuncture, using NaF (4 mg./ml.) as anti-coagulant. Glycerol can be determined in whole blood or in plasma. If sufficient blood is available, serum can

*) A unit is the amount of enzyme which catalyses the formation of 1 μmole of product per minute.

be used. Tissue samples must be removed as quickly as possible and frozen in liquid air. Blood and tissue extracts must be deproteinized. This preliminary treatment is unnecessary for urine, cerebrospinal and other body fluids.

Deproteinization

To 1 ml. of blood (plasma or serum) add 0.1 ml. perchloric acid solution (II) and after thorough mixing with a thin glass rod, centrifuge. Wash the precipitate with *ca.* 0.5 ml. 3% perchloric acid solution (III) and combine the washing with the first supernatant. Carefully adjust pH to about 9–9.5 with 10 N KOH and dilute with distilled water to 2.5 ml. (The use of graduated, conical centrifuge tubes is recommended.) After allowing to stand for 10 min. in an ice bath, quickly centrifuge off the $KClO_4$ precipitate and use the clear supernatant for the determination of glycerol.

A simpler procedure is to dilute 1 ml. plasma with 4 ml. distilled water and to heat for 5 min. in a boiling water bath. After centrifugation 0.5 ml. of the clear supernatant is analysed[7a].

To prepare deproteinized extracts of animal tissues, homogenize the tissues, which have been frozen in liquid air, with 4 volumes (4 ml./g. fresh weight of tissue) cold 3% perchloric acid solution (III) in a *Potter*[8] glass homogenizer or in a blendor. Treat the acid extract as described for blood.

Spectrophotometric measurements

Wavelength: 366 mμ; narrow cuvettes, 2 cm. light path; final volume: 2.0 ml.
Pipette the solutions in the following order:

Experimental cuvette

1.37 ml. buffer (solution I)

0.02 ml. GDH suspension (VI)

0.05 ml. ATP solution (IV)

0.05 ml. DPN solution (V)

0.50 ml. deproteinized sample.

Measure optical density E_1 against a cuvette containing water or against air. If E_1 is not constant, wait until the optical density change ceases or reaches a steady rate. Start the reaction by the addition of

0.01–0.02 ml. glycerokinase suspension (VII).

Follow the immediate increase in optical density until the end value E_2 is reached. Occasionally no steady end-point is obtained, so that E_2 must be calculated by extrapolation from the minimal rate of the slow optical density change (refer to p. 39). For such cases an automatic recording spectrophotometer is advantageous. The change in optical density due to the glycerokinase preparation can be obtained if on completion of the assay the same amount of enzyme is again pipetted into the cuvette. With highly purified or crystalline GK the absorption caused by the enzyme can be neglected. The optical density increase $\Delta E = E_2 - E_1$ is used for the calculations.

[7a] *L. Weiss*, personal communication.

[8] *V. R. Potter* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 10.

Calculations

For each mole of glycerol 1 mole of DPNH is formed. The extinction coefficient of DPNH at 366 mμ is $\varepsilon = 3.3$ cm.^{2}/μmole[9]. From the formula:

$$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles substance/assay mixture}$$

(V = final volume in the cuvette in ml., d = light path in cm.) it follows that

$$\frac{\Delta E_{366}}{3.3} = \mu\text{moles glycerol/assay mixture.}$$

If for example 0.5 ml. of deproteinized blood was taken, then $\Delta E_{366} \times 1.52 = \mu$moles glycerol/ml. blood (serum or plasma). To convert from μmoles/ml. to the usual clinical data of mg% multiply by 9.2.

Further Determinations

If L-(−)-glycerol-1-phosphate is also present in the sample (refer to p. 215) it can be determined as well as glycerol by following the optical density increase occurring on addition of GDH. L-(+)-Lactate can also be determined in the same assay mixture (refer to p. 266), providing that it is not present in too great an excess compared with the other two substrates. After completion of the glycerophosphate reaction crystalline lactic dehydrogenase (LDH, commercial preparation, see p. 986) is added and the increase in optical density recorded.

Sources of Error

It is essential to use LDH-free GDH for the determination of glycerol in blood. The reactions used to determine glycerol have the disadvantage, in common with all enzymatic estimations in which the equilibrium has to be displaced, of being slow to reach completion. The use of large amounts of enzyme for the assay is therefore recommended.

Specificity

Under the conditions described here the assay is specific for glycerol, since GDH only reacts with L-(−)-glycerol-1-phosphate. GK also phosphorylates dihydroxyacetone with ATP to give dihydroxyacetone phosphate[3]. Therefore it is possible to determine this compound enzymatically (see p. 244).

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[9] *H. J. Hohorst*, Biochem. Z. *328*, 509 [1957].