Hydroxypyruvate

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Principle

Hydroxypyruvate is reduced to L-glycerate by reduced diphosphopyridine nucleotide (DPNH) and lactic dehydrogenase from animal tissues[1]:

(1) Hydroxypyruvate + DPNH + H^+ $\rightleftharpoons$ L-glycerate + DPN^+

D-Glyceric dehydrogenase from plant tissues[1,2] catalyses a DPNH-dependent reduction of hydroxypyruvate to D-glycerate:

(2) Hydroxypyruvate + DPNH + H^+ $\rightleftharpoons$ D-glycerate + DPN^+

The equilibrium constant $K = [\text{hydroxypyruvate}] \times [\text{DPNH}]/[\text{D-glycerate}] \times [DPN^+]$ for reaction (2) was found to be 3×10^{-5} at pH 7.9 and 22°C[2,3]. Virtually the same constant applies to reaction (1), since reactions (1) and (2) differ only in the D and L configuration of the glycerate which plays no part in the energetics of the reaction. With a suitable excess of DPNH, hydroxypyruvate is almost quantitatively reduced to D or L-glycerate with the oxidation of 1 mole DPNH for each mole of hydroxypyruvate present. Hydroxypyruvate can be determined with lactic dehydrogenase according to equation (1) if the sample does not contain pyruvate. The determination is carried out in the same manner as the determination of pyruvate with lactic dehydrogenase (see p. 253). However, if hydroxypyruvate has to be estimated in the presence of pyruvate, the plant D-glyceric dehydrogenase must be used, since this enzyme does not reduce pyruvate. (Further details, see p. 262).

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Sodium hydroxide, A. R., 2 N
3. Hydrochloric acid, A. R., 1 N
4. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
5. D-Glyceric dehydrogenase
   preparation of the enzyme, see p. 262.

Preparation of Solutions

I. Tris buffer (0.1 M; pH 7.4):
Dissolve 4.86 g. of tris-hydroxymethyl-aminomethane in about 200 ml. doubly distilled water, add 33.6 ml. 1 N HCl and dilute with doubly distilled water to 400 ml. Bring temperature to 23°C and check pH with a glass electrode.

II. Reduced diphosphopyridine nucleotide (*ca.* 10^{-2} M β-DPNH):
Dissolve 10 mg. DPNH-Na_2 in 1 ml. doubly distilled water.

III. D-Glyceric dehydrogenase[2]:
Enzyme suspension in 1.5 M $(NH_4)_2SO_4$ solution, containing 2 to 5 mg. protein/ml. The specific activity should at least be 35000 units*/mg.[2].

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*) One unit is the amount of enzyme which reduces the optical density of DPNH by 0.001/min. at 366 mμ and 22–23°C, in a final volume of 3 ml. and with a 1 cm. light path.

1) *H. E. Stafford, A. Magaldi* and *B. Vennesland*, J. biol. Chemistry *207*, 621 [1954].
2) *H. Holzer* and *A. Holldorf*, Biochem. Z. *329*, 292 [1957].
3) *I. Zelitch*, J. biol. Chemistry *216*, 553 [1955].

Procedure

For preparation and extraction of the experimental material (blood, tissue, *etc.*), see determination of pyruvate with lactic dehydrogenase (p. 254).

Spectrophotometric measurements

Wavelength: 366 mμ; glass cuvettes, light path: 1 cm.; final volume: 3.00 ml.
A control cuvette is prepared differing from the experimental cuvette only in that it contains water instead of the sample to be analysed. Both these cuvettes are read against a water blank. Pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
1.50 ml. buffer (solution I)	1.50 ml. buffer (solution I)
0.05 ml. DPNH solution (II)	0.05 ml. DPNH solution (II) *)
Sample (containing 0.03—0.3 μmoles hydroxypyruvate)	water to give a total volume of 2.98 ml.
water to give a total volume of 2.98 ml.	

Read the optical densities of both cuvettes for a period of 2—3 min. If the change in optical density is not greater than 0.001 to 0.002 per 30 seconds then, using a glass spatula, mix

0.002 ml. enzyme suspension (III)

into both cuvettes with vigorous stirring to start the reaction. Read the optical densities until no further change occurs, or until the change is small and of the same magnitude in both cuvettes. As a rule this occurs after 4—6 min. The differences in the optical densities before addition of enzyme and after completion of the reaction are calculated for both cuvettes.

Experimental cuvette:

Optical density before addition of enzyme minus optical density on completion of reaction = ΔE_1

Control cuvette:

Optical density before addition of enzyme minus optical density on completion of reaction = ΔE_2

Subtract ΔE_2 from ΔE_1 to correct for unspecific changes in optical density due to the addition of the enzyme and any side reactions due to impurities, *etc.*:

$\Delta E_1 - \Delta E_2$ = change in optical density ΔE due to reduction of hydroxypyruvate.

Use ΔE for calculating the hydroxypyruvate concentration.

Calculations

$$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles hydroxypyruvate/cuvette}$$

ΔE = optical density change, V = cuvette contents in ml., d = light path in cm., ε = extinction coefficient for DPNH at 366 mμ (= 3.3 cm.²/μmole).

*) In order to obtain the same initial optical density in the control cuvette as in the experimental cuvette more DPNH can be added to compensate for the absorption of the sample containing hydroxypyruvate.

Other Determinations

The following metabolites can be determined in the same test system after the estimation of hydroxypyruvate: pyruvate by addition of lactic dehydrogenase, α-oxoglutarate[2] by addition of glutamic dehydrogenase, ketoses[4] by addition of polyol dehydrogenase, and other metabolites can be determined by addition of the corresponding enzymes (*e.g.* triosephosphate and dihydroxyacetone phosphate). However, it is necessary ensure that there is a sufficiently high concentration of DPNH present.

Specificity

D-Glyceric dehydrogenase from spinach leaves[2] reduces only hydroxypyruvate and glyoxylate. The reaction rate with glyoxylate is 4 to 5 times slower than that with hydroxypyruvate. On the other hand glyoxylic reductase from tobacco leaves[3] reduces glyoxylate 2 to 3 times more rapidly than hydroxypyruvate. No details are available for the specificity of D-glyceric dehydrogenase from parsley[5].

Appendix

Preparation of enzymes for the determination of hydroxypyruvate

The following enzyme preparations can be used for the reduction of hydroxypyruvate to D-glycerate.

1. A D-glyceric dehydrogenase of high purity can be obtained in 2–3 days from young spinach leaves, by ammonium sulphate fractionation, acid precipitation and adsorption on alumina-C_γ-gel[2].
2. A very active D-glyceric dehydrogenase can be obtained from frozen parsley leaves by ammonium sulphate fractionation. This preparation can be used without further purification[5].
3. A crystalline glyoxylate reductase, which also reduces hydroxypyruvate to D-glycerate[3], can be prepared from tobacco leaves by ammonium sulphate fractionation, treatment with protamine sulphate, and chromatography on calcium phosphate gel.

All 3 preparations are stable for months at −15° to −18°C. Pure hydroxypyruvate for assays of activity during the enzyme purification can be prepared from pyruvate by way of bromopyruvate[6] and isolation as the barium[7] or lithium salt[8].

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4) *H. Holzer* and *W. Goedde*, Biochim. biophysica Acta *40*, 297 [1960].
5) *F. Dickens* and *D. H. Williamson*, Biochem. J. *68*, 84 [1958].
6) *D. B. Sprinson* and *E. Chargaff*, J. biol. Chemistry *164*, 417 [1946].
7) *S. Akabori* and *K. Uehara* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 249.
8) *F. Dickens* and *D. H. Williamson*, Biochem. J. *68*, 74 [1958].