Inosine

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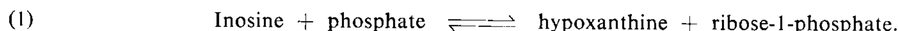
The method described here was developed by *Kalckar*[1]. It has only been used for the analysis of pure, aqueous solutions of inosine.

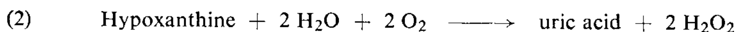
Principle

Nucleoside phosphorylase catalyses the reaction:



(1) Inosine + phosphate $\rightleftharpoons$ hypoxanthine + ribose-1-phosphate.

At pH 7.4 and in the presence of excess phosphate the reaction proceeds quantitatively from left to right. The hypoxanthine formed is then oxidized with xanthine oxidase to uric acid:



(2) Hypoxanthine + 2 H_2O + 2 O_2 $\longrightarrow$ uric acid + 2 H_2O_2

The increase of optical density at 293 mμ due to the formation of uric acid is a measure of the amount of inosine present.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 7\,H_2O$
3. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2\,H_2O$.
4. Inosine
   commercial preparation, see p. 1022.
5. Xanthine oxidase
   prepared according to[2] from milk; isolation, see p. 499.
6. Nucleoside phosphorylase
   prepared according to[3] from calf spleen; isolation, see p. 504.

Purity of the enzyme preparations

The preparations described on p. 499 and 504 satisfy the requirements.

Preparation of Solutions

I. Phosphate buffer (0.05 M; pH 7.4):
   a) Dissolve 27.2 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml.
   b) Dissolve 53.6 g. $Na_2HPO_4 \cdot 7\,H_2O$ in doubly distilled water and make up to 1000 ml.
   Mix 19.0 ml. solution a) with 81.0 ml. solution b) and dilute to 400 ml. with doubly distilled water.

II. Ethylene-diamine-tetra-acetate, EDTA (0.01 M):
   Dissolve 0.372 g. EDTA-$Na_2H_2 \cdot 2\,H_2O$ in doubly distilled water and make up to 100 ml.

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[1] *H. M. Kalckar*, J. biol. Chemistry *167*, 429 [1947].
[2] *H. Klenow* and *R. R. Emberland*, Arch. Biochem. Biophysics *58*, 276 [1955].
[3] *V. E. Price*, *M. C. Otey* and *P. Plesner* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. II, p. 448.

III. Inosine standard solution (5×10^{-3} M):
Dissolve 26.8 mg. inosine in doubly distilled water and make up to 20 ml.

IV. Nucleoside phosphorylase (129000 units*)/ml.):
Dilute the preparation obtained according to[3] with doubly distilled water.

V. Xanthine oxidase (14000 units**)/ml.):
Dilute the preparation obtained according to[2] or the commercial preparation with doubly distilled water.

Stability of the solutions

Store the enzyme solutions frozen at −17°C. They keep for at least a year. Repeated freezing and thawing leads to the customary loss of activity. Store all other solutions at 0°C and prepare freshly every three months.

Procedure

Experimental material

So far only pure, aqueous solutions of inosine have been analysed with the method described here.

Spectrophotometric measurements

Preliminary remarks: Allow the reagents to warm to room temperature before the assay. Thaw the enzyme solutions and keep in an ice bath.

Method: Wavelength: 293 mμ; light path: 1 cm.; final volume 3.0 ml. Measure against a control cuvette.

Pipette the solutions into the cuvettes in the following order:

Experimental cuvette	*Control cuvette*
2.700 ml. buffer (solution I)	2.700 ml. buffer (solution I)
0.030 ml. EDTA solution (II)	0.030 ml. EDTA solution (II)
0.020 ml. sample (containing 0.1 μmole inosine) or standard solution (III)	0.270 ml. doubly distilled water
0.210 ml. doubly distilled water	
0.035 ml.†) xanthine oxidase solution (V).	

Mix (blow air through the solution) and read the optical density at minute intervals for 5 min. until constant: E_1. Pipette into the experimental cuvette

0.005 ml. nucleoside phosphorylase solution (IV),

mix with a stream of air and read the optical density every 5 min. until constant: E_2.

The optical density of the nucleoside phosphorylase solution (IV) at 293 mμ must be determined separately: prepare two cuvettes similar to the control cuvette, mix into one cuvette 0.005 ml. nucleoside phosphorylase solution (IV) and measure the increase in optical density E_P against the enzyme-free cuvette. E_P is small and need only be determined once for each fresh nucleoside phosphorylase preparation.

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*) A unit is the amount of enzyme which increases the optical density at 293 mμ by 0.001 in 1 min. at 20°C with 1.5 μmoles inosine and excess xanthine oxidase in a 3 ml. reaction mixture (0.05 M phosphate buffer; pH 7.4; light path: 1 cm.).

**) Units as under*), but with hypoxanthine as the substrate.

†) = *ca.* 500 units.

Calculations

$$\frac{(E_2 - E_1 - E_P) \times 3}{12} = \mu\text{moles inosine/reaction mixture}$$

where

E_2 = optical density after the addition of nucleoside phosphorylase
E_1 = optical density before the addition of nucleoside phosphorylase
E_P = optical density of the nucleoside phosphorylase solution
3 = assay volume [ml.]
12 = extinction coefficient at 293 mμ for the conversion of hypoxanthine → uric acid [cm.²/μmole]

Appendix

Isolation of nucleoside phosphorylase[3]

The procedure comprises the following steps: extraction of calf spleen with water; precipitation of inactive protein at pH 5.2 (acetate buffer); precipitation of active protein with ethanol (20%) at −5° C; fractionation by repeated homogenization of the precipitate with ethanol-acetate buffer and glycine-acetate buffer; precipitation of inactive protein with $(NH_4)_2SO_4$.