Adenosine

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The spectrophotometric determination of purine compounds with specific enzymes was described in detail by *Kalckar*[1,2] in 1947. The quantitative enzymatic determination of adenosine with adenosine deaminase from intestinal mucous membrane has been widely used because of the high specificity of the enzyme.

Principle

Adenosine deaminase catalyses the deamination of adenosine to inosine:

(1) $$\text{Adenosine} + H_2O \rightleftharpoons \text{inosine} + NH_3$$

The equilibrium lies far to the right. The reaction goes to completion in a few minutes at room temperature in phosphate buffer (pH 7.4)[3]. As the absorption maximum of adenosine is at 265 mμ and that of inosine is at 247 mμ[4], the decrease of optical density at 265 mμ is a measure of the reaction.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Dipotassium hydrogen phosphate, K_2HPO_4
3. Adenosine deaminase
   from calf small intestine[2], suspension in 2.5 M ammonium sulphate solution; commercial preparation, see p. 968.

For deproteinization:

4. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
5. Potassium carbonate, K_2CO_3, A. R.

Purity of the enzyme preparation

If the assay is carried out in phosphate buffer, then a crude enzyme preparation (5 – 10 units *)/mg.) is satisfactory; it may contain phosphatases, because in the presence of phosphate interfering phosphatases are completely inhibited[3]. Otherwise the specific activity should be >100 units *)/mg. and the preparation must not contain more than 0.01% phosphatase (relative to the specific activity of the deaminase).

Preparation of Solutions (for *ca.* 20 determinations)

I. Phosphate buffer (0.05 M; pH 7.4):
Mix 19 ml. of a solution of 680 mg. KH_2PO_4/100 ml. doubly distilled water with 81 ml. of a solution of 870 mg. K_2HPO_4/100 ml. doubly distilled water.

II. Adenosine deaminase (100 μg. protein/ml.):
Dilute the enzyme suspension with 2.5 M ammonium sulphate solution.

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*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min.[5].

1) *H. M. Kalckar*, J. biol. Chemistry *167*, 429 [1947].
2) *H. M. Kalckar*, J. biol. Chemistry *167*, 445 [1947].
3) *A. Kornberg* and *W. E. Pricer* jr., J. biol. Chemistry *193*, 481 [1951].
4) *J. M. Gulland* and *E. R. Holiday*, J. chem. Soc. [London] *1936*, 765.
5) *J. Cooper, P. A. Srere, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].

For deproteinization:

III. Perchloric acid (1 M):
Dilute 9 ml. 70% $HClO_4$ (sp. gr. 1.67) with doubly distilled water to 100 ml.

IV. Potassium carbonate (*ca.* 5 M):
Dissolve 69 g. K_2CO_3 in doubly distilled water and make up to 100 ml.

Stability of the solutions

Store the enzyme and buffer solution, stoppered, at 0 to 4° C. Both are stable for several months as long as no bacterial contamination occurs.

Procedure

Experimental material

Samples containing small amounts of protein need not be deproteinized. This includes nucleic acid hydrolysates, mixtures of nucleotides and nucleosides, adenosine preparations and preparations which have to be assayed for contamination by adenosine. Usually the measurements must be made against a blank (with sample, but no enzyme) to compensate for the optical density due to other purine and pyrimidine derivatives.

Deproteinize samples containing large amounts of protein (*e.g.* yeast concentrates) with perchloric acid[1, 2]. Trichloroacetic acid is unsuitable, because it absorbs strongly in the UV region.

Deproteinization

Pipette into a 10 ml. centrifuge tube:

1 volume perchloric acid solution (III)
1 volume sample

Mix thoroughly with a thin glass rod, centrifuge for 5 min. (*ca.* 3000 g), neutralize 1 ml. of the supernatant with

ca. 0.05 ml. K_2CO_3 solution (IV).

Allow to stand for 10 min. in an ice bath, filter and use 0.5 ml. of the filtrate for the assay.

Spectrophotometric measurements

Preliminary remarks: If the sample contains large amounts of other nucleosides and nucleotides which have a high absorption at 265 mμ and therefore make the accurate determination of adenosine difficult, it is necessary to determine the approximate optical density change due to the adenosine in a preliminary assay. Then in the actual determination, measurements are made against a blank, which contains sufficient sample to compensate for at least 3/4 of the optical density at 265 mμ not due to adenosine.

Method: Wavelength: 265 mμ; silica cuvettes, light path: 1 cm.; final volume: 3 ml.; room temperature. Measure against a blank cuvette containing buffer solution (I) or buffer solution + sample (see above).

Pipette successively into the cuvette

2.48 ml. buffer (solution I)
0.50 ml. sample.

Mix and read the initial optical density E_1 (should not be greater than *ca.* 0.500). Mix in

0.02 ml. adenosine deaminase suspension (II)

with a small glass or plastic rod flattened at one end. Read the optical density every minute and after 5—10 min. read the final value E_2. To determine the absorption due to the enzyme again mix in

0.02 ml. adenosine deaminase suspension (II)

and read the optical density E_3 (only necessary with crude enzyme preparations containing <100 units/mg.). Subtract $E_3 - E_2 = \Delta E_E$ from E_2. $\Delta E = E_1 - (E_2 - \Delta E_E)$ is used for the calculations.

Calculations

$\Delta E = E_1 - (E_2 - \Delta E_E)$ is proportional to the amount of adenosine. Standard curves are linear and pass through the origin; we found with absolutely pure adenosine an optical density difference of $\Delta E = 0.303$ instead of the value 0.263 [2] for 10 μg. adenosine/ml. It follows that $\Delta E = 0.0101$ for 1 μg. adenosine in a final volume of 3 ml. Therefore:

$$\frac{\Delta E}{0.0101} = \mu g.\ \text{adenosine/0.5 ml. sample}$$

To convert to μg. adenosine/ml. sample it is necessary to divide by the volume of sample taken for the assay.

If the sample was deproteinized then it is also necessary to multiply by the dilution factor (2 + ml. K_2CO_3 solution).

Sensitivity: If a range of the photometer scale is chosen where $\Delta E = 0.010$ can be read with sufficient accuracy, then it is possible to determine as little as 2 μg. adenosine/ml. sample.

Example

A nucleic acid hydrolysate was diluted 1 : 30 with doubly distilled water; 0.5 ml. was taken for the assay. Measurements were made with a crude enzyme preparation. In a preliminary assay it was found that $E_1 \approx 1.5$, $E_2 \approx 1.3$. The optical density at 265 mμ not due to adenosine was 1.3. In the actual determination a blank containing $\frac{3}{4} \times 1.3 \times \frac{0.5}{1.5}$ *) ≈ 0.33 ml. sample was prepared.

Measurements were made against this blank (time of reaction: 5 min.): $E_1 = 0.503$; $E_2 = 0.319$; $E_3 = 0.325$; $\Delta E_E = E_3 - E_2 = 0.006$; $E_2 - \Delta E_E = 0.319 - 0.006 = 0.313$; $\Delta E = 0.503 - 0.313 = 0.190$.

$$\frac{0.190}{0.0101} \times \frac{1}{0.5} \times 30 = 1130\ \mu g.\ \text{adenosine/ml. sample}$$

0.5 = volume of sample in the assay mixture [ml.]
30 = dilution factor.

Other Determinations

If the crude adenosine deaminase preparations contain alkaline phosphatases and pyrophosphatases, or if these enzymes are added to the assay system, then all compounds which contain adenosine phosphate react (*e.g* A-2′-MP, A-3′-MP, A-5′-MP, CoA, DPN, TPN). By the successive stepwise addition

*) $\frac{0.5}{1.5}$ = conversion of E_1 in the preliminary assay (1.5) to the value of $E_1 \approx 0.5$ aimed at in the actual determination.

of phosphatases it is possible to distinguish between adenosine, adenosine phosphates and their derivates [2,6–8].

Sources of Error

1. Occasionally tissue filtrates contain inhibitors of adenosine deaminase. If the deamination proceeds slowly or not at all, then first add more enzyme. If this is without success, then test whether the assay system is functioning correctly by the addition of *ca.* 5 μg. pure adenosine. Recovery of the added adenosine within a few minutes indicates that inhibitors are absent. Otherwise the sample must be purified with an ion exchange resin (*e.g.* Amberlite IR 4 B or Dowex 1 X 10).
2. Turbidity of any kind produces a high optical density at 265 mμ; it must be avoided. This also includes precipitates which are formed from constituents of the sample (*e.g.* Mg^{2+}) and the phosphate buffer.

Specificity

The enzyme deaminates adenosine to inosine and 2,6-diaminoribofuranosylpurine to guanosine *). Adenosine deaminase does not react with deoxyriboadenosine, riboguanosine, ribocytidine, A-5′-MP, A-3′-MP, A-2′-MP, ATP or adenine [8,9].

*) The reaction product, inosine, can be distinguished from guanosine by enzymatic determination with nucleosidase and xanthine oxidase (see p. 502).

6) *T. P. Wang, L. Shuster* and *N. O. Kaplan*, J. biol. Chemistry *206*, 299 [1954].
7) *L. Shuster, N. O. Kaplan* and *F. E. Stolzenbach*, J. biol. Chemistry *215*, 195 [1955].
8) *N. O. Kaplan* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. II, p. 473.
9) *G. Schmidt* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 781.