Adenosine Phosphates

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The method described here determines A-2′-MP, A-3′-MP, A-5′-MP, adenosine-2′,5′-diphosphate, adenosine-3′,5′-diphosphate and the cyclic adenosine phosphates. A-5′-MP can be determined separately (*e.g.* with myokinase, see p. 573). The determination of adenosine-2′ and 3′ phosphates is of importance in the isolation of these compounds from mixtures of nucleotides. *Kalckar*[1] was the first to describe a suitable method requiring alkaline phosphatase and adenosine deaminase.

Principle

Alkaline phosphatase hydrolyses adenosine phosphates to adenosine and inorganic phosphate. Adenosine deaminase deaminates adenosine to inosine (see determination of "Adenosine", p. 491), causing the optical density at 265 mμ to decrease by an amount corresponding to the amount of adenosine present.

Reagents

1. Triethanolamine hydrochloride
2. Sodium hydroxide, A. R., 0.1 N
3. Alkaline phosphatase
   from intestinal mucous membrane, dry powder. Commercial preparation, see p. 991.
4. Adenosine deaminase
   from intestinal mucous membrane, suspension in 2.5 M ammonium sulphate solution. Commercial preparation, see p. 968.

Purity of the enzyme preparations

If the sample does not contain adenosine, then a crude adenosine deaminase preparation is satisfactory since this is usually contaminated with sufficient phosphatase activity. For the procedure described here the deaminase should contain $<0.01\%$ phosphatase (relative to the specific activity of the deaminase) and should have a specific activity of >100 units*)/mg. The phosphatase may be contaminated with deaminase, but should contain $<0.01\%$ pyrophosphatase (relative to the specific activity of the phosphatase) if pyrophosphate-containing dinucleotides (DPN, TPN, CoA) are present in the sample; the specific activity should be *ca.* 50 units*)/mg.

Preparation of Solutions

I. Triethanolamine buffer (0.05 M; pH 8.4):
   Dissolve 930 mg. triethanolamine hydrochloride in doubly distilled water, make up to 60 ml. and add 40 ml. 0.1 N NaOH.

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

III. Phosphatase (5 mg. protein/ml.):
   Dissolve 5 mg. dry powder in 1 ml. triethanolamine buffer (solution I). If necessary, centrifuge turbid solutions at high speed to clarify.

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*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min.[2].

1) *H. M. Kalckar*, J. biol. Chemistry *167*, 445 [1947].
2) *J. Cooper, P. A. Srere, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].

Stability of the solutions

Store all solutions at 0 to 4° C. The buffer solution and deaminase suspension keep for several months. Prepare the phosphatase solution freshly each week. The phosphatase can be stored for several months as a dry powder at 0 to 4° C.

Procedure

Experimental material

Protein must be removed from samples since it absorbs at 265 mμ (see p. 492). Nucleotide mixtures, nucleic acid hydrolysates and similar material need not be deproteinized.

Spectrophotometric measurements

Preliminary remarks: The samples usually contain other nucleotides and nucleosides which absorb strongly at 265 mμ and therefore prevent the accurate determination of AMP and adenosine. Prepare a blank cuvette to compensate for this (see "Adenosine", p. 491).

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

Pipette successively into the cuvette:

2.46—2.94 ml. buffer (solution I)
0.50—0.02 ml. sample.

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

0.02 ml. deaminase suspension (II)

with a small glass or plastic rod flattened at one end (the optical density of the enzyme is negligible). Read the optical density at 1 min. intervals and after *ca.* 5 min. measure the final optical density E_2. Then mix in

0.02 ml. phosphatase solution (III)

and read the optical density at 1 min. intervals until the final value E_3 is reached (5—15 min.). Again mix in

0.02 ml. phosphatase solution (III)

and read the optical density E_4. $E_4 - E_3 = \Delta E_{Ph}$ is the optical density due to the phosphatase preparation; subtract this value from E_3. $E_2 - E_{3(corr.)} = \Delta E_{AMP}$ and $E_1 - E_2 = \Delta E_{adenosine}$ are used for the calculations.

ΔE_{AMP} represents the total amount of pyrophosphate-free adenosine phosphates present in the sample.

Calculations

The deamination of 1 μg. adenosine ($^1/_{267}$ μmole) in a final volume of 3 ml. results in an optical density change at 265 mμ of $\Delta E = 0.0101$ (p. 493).

Therefore:

$$\frac{\Delta E_{adenosine}}{0.0101 \times (\text{ml. sample in assay})} = \mu\text{g. adenosine/ml. sample}$$

$$\frac{\Delta E_{adenosine}}{2.70 \times (\text{ml. sample in assay})} = \mu\text{moles adenosine/ml. sample}$$

$$\frac{\Delta E_{AMP}}{2.70 \times (\text{ml. sample in assay})} = \mu\text{moles adenosine phosphates/ml. sample}$$

$$\frac{\Delta E_{AMP}}{0.00777 \times (\text{ml. sample in assay})} = \mu\text{g. adenosine phosphates/ml. sample}$$

Sensitivity: If a range of the photometer scale is chosen on which $\Delta E = 0.010$ can be read with sufficient accuracy, then it is possible to determine as little as *ca.* 2 μg. (*ca.* 2×10^{-2} μmoles) adenosine or adenosine phosphates/ml. sample.

Example

The course of a hydrolysis of 3′(2′)-nucleotides to the corresponding nucleosides was controlled enzymatically. A 1 ml. sample of the hydrolysis reaction mixture was diluted 1 : 10 with water and 0.05 ml. was taken for the assay. In a preliminary assay (see p. 492) the following optical densities were obtained after the addition of the two enzymes: $E_1 \approx 1.2$, $E_3 \approx 0.8$. In the actual determination a blank was prepared which contained: $\frac{3}{4} \times 0.8 \times \frac{0.05}{1.2} = 0.025 \approx 0.03$ ml. sample (refer to p. 492).

The measurements were made against this blank:
$E_1 = 0.495$; $E_2 = 0.283$; $E_3 = 0.031$; $E_4 = 0.074$; $E_1 - E_2 = \Delta E_{adenosine} = 0.212$; $E_4 - E_3 = \Delta E_{Ph} = 0.043$; $E_3 - \Delta E_{Ph} = E_{3(corr.)} = -0.012$; $E_2 - E_{3(corr.)} = \Delta E_{AMP} = 0.283 - (-0.012) = 0.295$. It follows that adenosine content of the sample is:

$$\frac{0.212 \times 10}{2.70 \times 0.05} = 15.7 \text{ μmoles/ml. sample}$$

and the adenosine phosphate content is:

$$\frac{0.295 \times 10}{2.70 \times 0.05} = 21.8 \text{ μmoles/ml. sample.}$$

A-5′-MP could not be detected in the assay with myokinase (see p. 573).

Other Determinations

According to *Kalckar*[1] it is possible by the addition of adenylpyrophosphatase to estimate DPN, TPN and CoA in a third reaction step.

Sources of Error

See "Adenosine", p. 494 and under "Purity of the enzyme preparations".

Specificity

See "Adenosine". Only adenosine phosphates react.