A SPECTROPHOTOMETRIC ASSAY FOR RIBONUCLEASE ACTIVITY USING CYTIDYLYL–(3′,5′)–ADENOSINE AND URIDYLYL–(3′,5′)–ADENOSINE AS SUBSTRATES

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1. Introduction

Ribonuclease activity is usually measured by three general types of assay methods. A) the Kunitz spectrophotometric assay [1], in which the depolymerization of RNA is followed from the increase in absorption at 300 mμ; B) the precipitation assay, where the production of acid soluble fragments of RNA is measured at 260 mμ [2]; C) the cyclic phosphate hydrolase assay, based on the property of ribonuclease to hydrolyze pyrimidine 2′, 3′-cyclic phosphates [3]. The choice of the most advantageous procedure will depend on the particular experimental situation, and has been discussed by Klee [4].

Ribonuclease classically is considered to hydrolyze only those phosphodiester bonds of RNA that involve phosphate esterified to the 3′-hydroxyl of a pyrimidine nucleoside [5]. Thus cytidylyl–(3′,5′)–adenosine (CpA) and uridylyl–(3′,5′)–adenosine (UpA) are cleaved to give 3′-CMP and adenosine, and 3′-UMP and adenosine respectively.

The enzyme activity can therefore be measured from the amount of adenosine released from CpA or UpA, by a sensitive coupled optical assay, described herein. The adenosine formed is determined spectrophotometrically, after addition of an excess of adenosine deaminase.

2. Materials and methods

Type 1-A ribonuclease from bovine pancreas (5 X crystallized) was obtained from Sigma Chemical Company. CpA was purchased from Aldrich Chemical Company, and UpA from Zellstofffabrik Waldhof. Adenosine deaminase from intestinal mucosa (10 mg/ml stabilized solution), free of any detectable deaminating activity on CpA and UpA, was obtained from Boehringer und Soehne, and was diluted 500 fold with water before use.

The experiments were carried out at room temperature in 1 ml 1 cm path cuvets, in a final volume of 0.750 ml, and the change in optical density at 265 mμ was determined with a recording Zeiss PMQ II spectrophotometer.

The detailed standard procedure for the coupled optical enzyme assay was as follows: 0.5 ml of 0.1 M acetate buffer, pH 5, was pipetted into one cuvet, followed by different amounts of aqueous solutions of CpA or UpA, enough water to bring the volume to 0.700 ml, and 30 μl of the diluted solution of adenosine deaminase; the mixture was rapidly mixed and the optical density at 265 mμ was recorded against a reference cuvet, in which the substrate was substituted with water. Finally 20 μl of ribonuclease solution was added in both cuvets, and the decrease in optical density was recorded.

3. Results

Fig. 1 shows the results of some typical experiments. It can be seen that, in the absence of ribonuclease, the optical density at 265 mμ remained constant, showing the absence of ribonuclease activity in the auxiliary enzyme.

No change in the rate of the decrease in optical density was observed, at concentrations of adenosine

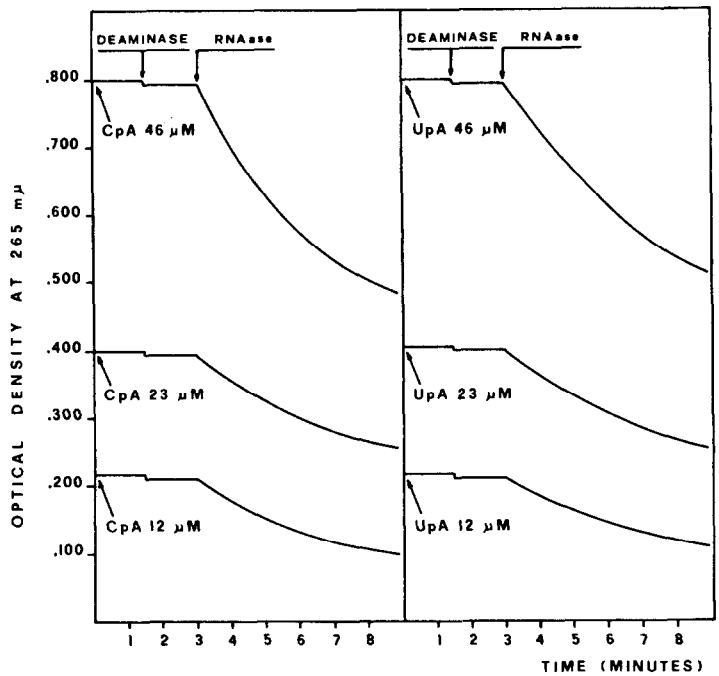

Fig.1. Time course of the change in optical density at 265 mμ during coupled optical test for ribonuclease at room temperature. Reaction mixture as described in the text, with the indicated final concentrations of CpA and UpA. 0.136 μg of ribonuclease per reaction mixture were used with CpA as substrate, and 0.068 μg with UpA as substrate.

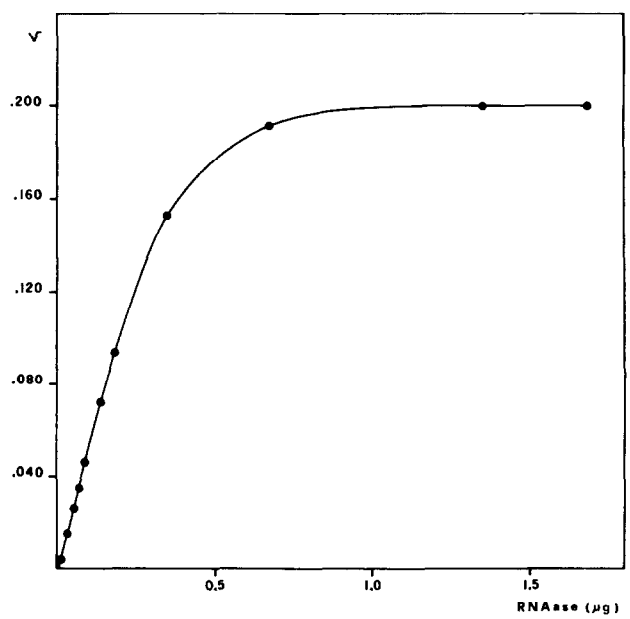

Fig.2. Enzyme activity as a function of ribonuclease concentration. The standard assay procedure was used. The substrate was 46 μM CpA. The velocity is expressed as ΔE/min at 265 mμ.

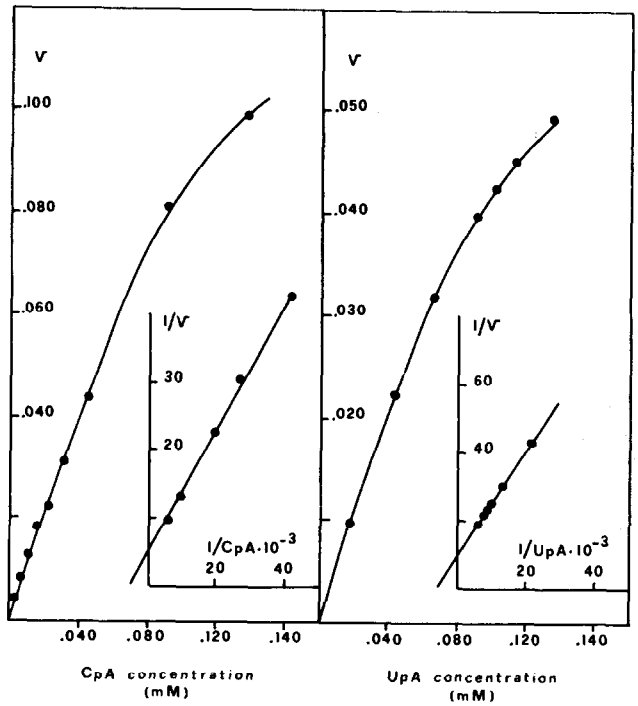

Fig.3. Effect of CpA and UpA concentration on the velocity of ribonuclease. The insets show the Lineweaver and Burk [6] plots for the determination of the K_m for CpA and UpA. The velocity is expressed as in fig. 2.

deaminase considerably lower or higher than those used in the standard assay; the velocity of the reaction is therefore determined by the rate at which adenosine is released from CpA or UpA. Furthermore the two substrates used had no inhibitory effect on adenosine deaminase.

Fig. 2 shows the rate of optical density change at 265 mμ during coupled optical density test as a function of ribonuclease concentration.

The evaluation of the K_m for CpA and UpA by the use of double reciprocal plots is shown in fig. 3. It is interesting to note that for both substrates a K_m value of 0.27 mM was obtained.

In our hands the method described above proved to be a rapid and accurate assay. For its sensitivity and simplicity it lends itself well for kinetic studies and for many routine applications. In principle the method can also be used to investigate the specificity of different ribonucleases, using other commercially available dinucleoside-monophosphates, such as guanylyl-(3′,5′)-adenosine and adenylyl-(3′,5′)-adenosine as substrates.

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