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Polarimetric assay of the activity of ribonuclease on cyclic nucleotides*

Various methods have been described for following the hydrolysis of pyrimidine 2′,3′-cyclic phosphates by ribonuclease (ribonucleate pyrimidinenucleotido-2′-transferase (cyclizing), EC 2.7.7.16)[1–3]. These methods are based either on the titration, at constant pH, of the acidic groups released during hydrolysis, or on measuring the change in absorbance at a selected wavelength accompanying the reaction. The titration method suffers from the disadvantage that the ionic strength of the solution and the concentrations of the reactants are continuously changing during the assay. In the spectrophotometric method, the high extinction coefficients of the substrate and the product limit the range of substrate concentration at which the assay can be performed.

In this communication, a polarimetric method is described for the assay of the enzymic hydrolysis of cytidine 2′,3′-cyclic phosphate. The conversion of this cyclic phosphate into cytidylic acid is accompanied by a change of about 100 degrees·cm^2·$dmole^{-1}$ in the molar optical rotation at the sodium D line (24°). Following this change in optical rotation has been found to offer a convenient method for assaying the hydrolytic activity of pancreatic ribonuclease towards cytidine 2′,3′-cyclic phosphate over a wider range of substrate and enzyme concentrations than is possible by the other methods available.

Ribonuclease A from bovine pancreas (Sigma Chemical Co., 5 times recrystallized, type I-A, Lot No. 114B-1510) was used throughout. Enzyme concentrations were determined spectrophotometrically at 277.5 mμ (ref. 4), ε(277.5 mμ) = 9800 $l \cdot mole^{-1} \cdot cm^{-1}$. Solutions of cytidine 2′,3′-cyclic phosphate, sodium salt (Sigma Chemical Co., Lot No. 84B-0560), were kept frozen until just before use; their concentrations were determined spectrophotometrically at 268 mμ (ref. 5), ε(268 mμ) = 8170 $l \cdot mole^{-1} \cdot cm^{-1}$. Reagents used for the preparation of buffers were of analytical grade.

A Bendix-Ericsson polarimeter, ETL-NPL type 143 A, was used. Traces of the change in optical rotation with time were obtained by a Honeywell recorder, attached to the polarimeter. A resistance-capacitance electrical filter with a time constant of about 30 sec (47 000 Ω, 600 μF) was introduced at the recorder input; by this device the noise level of the polarimeter was readily reduced. Thus, the recorded random fluctuations did not exceed 0.0003° in optical rotation. The yellow light filter of the polarimeter, corresponding to the sodium D line, was used throughout.

Polarimeter tubes of 50-mm optical path were employed. The tube diameter was either 2.0 or 8.0 mm, the volume of solution required being about 0.5 and 5.0 ml, respectively. Less noise interference was experienced when the wider tubes were used. When the narrow tubes were used, the zero setting varied from one experiment to another, though it was stable during every experiment. The zero setting seemed to depend on the orientation of the tube in the polarimeter.

pH measurements were carried out with a Radiometer TTT1 pH-meter. A Zeiss PMQ II spectrophotometer was used for determinations of absorbances.

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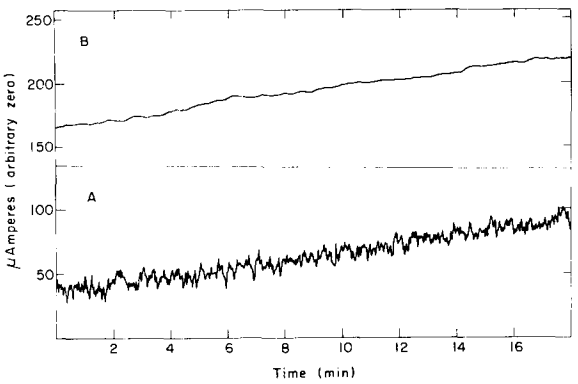
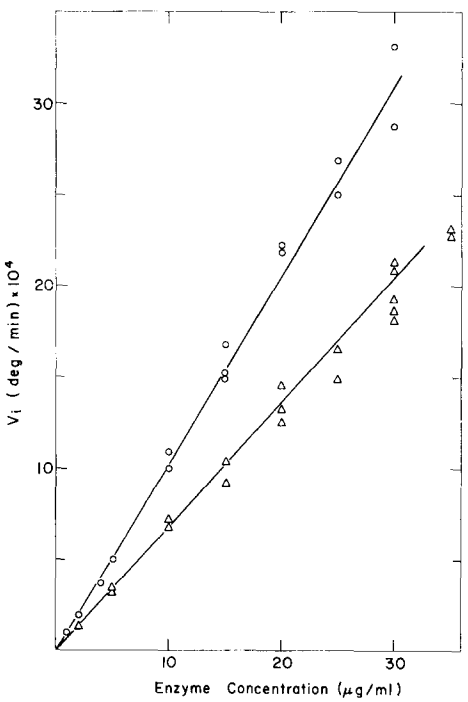


Fig. 1. Recorder trace of the change in optical rotation with time upon the hydrolysis of cytidine 2′,3′-cyclic phosphate by ribonuclease. Enzyme concentration, 3.0 μg/ml; substrate concentration, 1.85 mg/ml, pH 7.1 (0.1 M Tris–HCl, adjusted to $I = 0.2$ with NaCl); temp., 24°; polarimeter tube diameter, 8.0 mm. 1 μA corresponds to $6.28 \cdot 10^{-5}$ degrees of optical rotation at the sodium D line. A, recorder connected directly to polarimeter; B, electrical filter of 30-sec time constant inserted at recorder input.



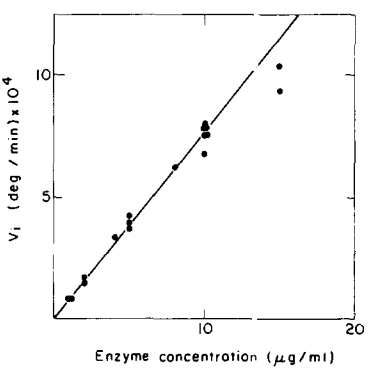


Fig. 2. Initial rates of change in optical rotation at the sodium D line, V_i, accompanying the enzymic hydrolysis of cytidine 2′,3′-cyclic phosphate at various ribonuclease concentrations. Substrate concentrations: ○, 3.7 mg/ml; △, 1.85 mg/ml. Temp., 24°; polarimeter tube diameter, 8.0 mm.

Fig. 3. Initial rates of change in optical rotation at the sodium D line, V_i, accompanying the enzymic hydrolysis of cytidine 2′,3′-cyclic phosphate at various ribonuclease concentrations. Substrate concentration, 1.85 mg/ml; temp., 24°; polarimeter tube diameter, 2.0 mm.

The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.1) and was adjusted to 0.2 ionic strength with NaCl. Activity assays were carried out at room temperature (24°). Substrate concentrations ranged between 2 and 4 mg/ml. Enzyme activities were conveniently expressed by the initial slopes of the traces of optical rotation *versus* time.

Fig. 1 illustrates a typical trace of the change in optical rotation with time upon the hydrolysis of cytidine 2′, 3′-cyclic phosphate by ribonuclease. Enzyme concentration was 3.0 μg/ml, substrate concentration was 1.85 mg/ml and the polarimeter tube was 8.0 mm in diameter. As may be seen from Fig. 1, the insertion of the electrical filter at the recorder input markedly reduces the noise level and renders the estimation of reaction rates more accurate and convenient.

In Fig. 2, the initial rates of change of optical rotation are plotted as a function of concentration of ribonuclease. Two different concentrations of substrate have been used: 3.7 and 1.85 mg/ml. Polarimeter tube diameter was 8.0 mm in these experiments. As is seen from the figure, the initial rates vary linearly with enzyme concentration within the range of 1 to 35 μg/ml. The scatter of the results increases somewhat at enzyme concentrations exceeding 20 μg/ml, but even then the scatter is within 10% of the measurements.

The above experiments were repeated with a polarimeter tube 2.0 mm in diameter and substrate concentration of 1.85 mg/ml. The results are shown in Fig. 3. In this case the measured initial rates vary linearly with enzyme concentration within the range of 1 to 10 μg/ml. At enzyme concentrations exceeding 15 μg/ml, the rate of hydrolysis is too fast for the initial rates to be reliably estimated when the narrower tubes were used. The relative precision of the assays when the 2.0-mm tubes are used is about 10%.

It is pertinent to point out in conclusion that the accuracy and sensitivity of the polarimetric method for the assay of ribonuclease activity can probably be increased if a polarimeter is available for measuring optical rotations in the ultraviolet region. Thus, the change in the molar optical rotation accompanying the hydrolysis of cytidine 2′,3′-cyclic phosphate was found to be about 10 times as large at 300 mμ as at the sodium D line.

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