Ribonuclease

Nepomuk Zöllner and Gerd Hobom

Ribonucleases occur in most animal tissues[1]. The best characterized is the ribonuclease from pancreas[2], which is also known as ribonuclease I. It is a specific phosphodiesterase, which liberates the pyrimidine nucleotides contained in ribonucleic acid as mononucleotides[3,4]; purine nucleotides remain in di-, tri-, and tetra-nucleotides, which always contain a terminal pyrimidine nucleotide[5]. Ribonuclease splits the ester linkage between a 3′-pyrimidine dinucleotide phosphate and the 5′-hydroxy group of an adjacent nucleotide, resulting in the formation of 3′-pyrimidine nucleotides. Intermediate products are cyclic 2′,3′-pyrimidine nucleotides. Pancreatic ribonuclease also hydrolyses the benzyl, methyl and ethyl esters of 3′-pyrimidine nucleotides[6]. With a high alcohol concentration it catalyses the synthesis of esters from cyclic nucleotides and alcohols[7]. Deoxyribonucleic acid, phenylphosphate, the phosphodiester of glycerol and the esters of 5′-pyrimidine nucleotides are not attacked. Some reactions of this ribonuclease have been described, however, which do not appear to fit in with the specificity described above[8,9].

Other ribonucleases, whose specificity differs from that of pancreatic ribonuclease, are known[8,10]. They are usually thermolabile and therefore can be easily differentiated from ribonuclease I. Ribonuclease can also be distinguished from unspecific phosphatases by its heat stability between pH 2.5 and 4.5. A heat stable ribonuclease occurs in tissue extracts[11], which in its stability to sulphuric acid and the effect on it of salts and buffers, is similar to pancreatic ribonuclease[12] and probably has similar catalytic properties[10].

During the hydrolysis of ribonucleic acid by pancreatic ribonuclease, products are formed which are not precipitated by acid. The acid groups of these can be determined titrimetrically, or manometrically. They can also be determined by measurement of their spectra, which in comparison to that of ribonucleic acid, are displaced towards the shorter wavelengths[13]. Ribonuclease has also been determined by the hydrolysis of cyclic pyrimidine nucleotides[14].

Methods in which the formation of acid-soluble, break-down products is determined are difficult to evaluate in practice, because the amount of these products is not proportional to the number of bonds hydrolysed at any time, and also ribonuclease is stable to acid[15]. The optimum conditions for stopping the enzyme reaction depend on the source of the ribonuclease preparation, therefore many methods are difficult to reproduce. On the other hand, these methods permit the determination of very small amounts of enzyme with high substrate concentration and long incubation times. Examples of the methods are: measurement[2] of the organically bound phosphate, which is not precipi-

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tated by *MacFadyens* reagent (uranyl acetate in trichloroacetic acid); measurement with orcinol[16] of the increase in acetone-HCl soluble nucleotides; direct spectrophotometric measurements at 260 mμ on the solution after precipitation with *Schencks* reagent ($HgCl_2$ in HCl) and removal of the mercury with H_2S[15].

The methods described here are the spectrophotometric method of *Kunitz*[13] (which is also the basis of a method for the determination of heparin[17], see p. 79) and a titrimetric method[18].

A. Spectrophotometric Method[13]

Principle

The action of ribonuclease on ribonucleic acid results in a decrease in the optical density at 300 mμ.

Reagents

1. Acetic acid, glacial
2. Sodium acetate, A. R., anhydrous
3. Ribonucleic acid
   from yeast, sodium salt; commercial preparation, see p. 1027.
4. Ribonuclease, RNase
   from pancreas, crystalline; commercial preparation, see p. 997.

Preparation of Solutions

Prepare all solutions with doubly glass distilled water.

I. Acetate buffer (0.1 M; pH 5.0):
Mix 70.5 parts 0.1 M sodium acetate solution (8.2 g/1000 ml.) and 29.5 parts 0.1 N acetic acid (5.7 ml. acetic acid/1000 ml.).

II. Acetate buffer (0.2 M; pH 3.7):
Mix 1 part 0.2 M sodium acetate solution (16.4 g./1000 ml.) and 9 parts 0.2 N acetic acid (11.5 ml. acetic acid/1000 ml.).

III. Ribonucleate (0.1% w/v):
Dissolve 0.1 g. sodium ribonucleate in 100 ml. 0.1 M acetate buffer (solution I).

IV. Ribonuclease, RNase (50 μg. protein/ml.):
Dissolve 5 mg. crystalline ribonuclease from pancreas in 100 ml. doubly distilled water.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator at 2 to 4°C. The substrate solution (III) keeps in a refrigerator for about 2 months if a crystal of thymol is added. The ribonuclease solution (IV) loses about 30% of its activity in two months.

Procedure

Experimental material

Before the determination destroy unspecific phosphatases by heating at acid pH. To do this, mix the sample (serum, secretions, urine) with an equal volume of acetate buffer (solution II) and place for 10 min. in a boiling water bath. Centrifuge off the precipitate.

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Spectrophotometric measurements

Wavelength: 300 mμ; light path: 1 cm.; final volume: 2 ml.; room temperature, should be constant for a series of measurements. Before starting the measurements equilibrate all the solutions (water bath); for measurements at higher temperatures use a constant temperature cuvette holder. Measure against a control cuvette containing water; zero instrument with this.

Pipette successively into the cuvette:

2 ml. ribonucleate solution (III)
2 ml. sample.

Mix quickly, start a stopwatch and read the optical density (E_0) within 10 sec. Continue to read the optical densities E_t at 15, 30 and 60 sec. and then every minute for a total of 10 min. Once for each ribonucleate preparation continue to read at longer time intervals until the reaction stops (1 to 3 hours). Read the final optical density E_f.

Calculations

General information: The rate of the reaction is given by:

$$(1) \qquad -\frac{dE}{dt} = kc\,(E_t - E_f)$$

where c = concentration of ribonuclease
k = rate constant

At any time the rate is proportional to the amount of the unhydrolysed substrate. This is equivalent to the optical density difference $E_t - E_f$ (E_t = optical density at time t; E_f = optical density at the end of the reaction). Integration of equation (1) gives:

$$(2) \qquad -\log (E_t - E_f) = \frac{kct}{2.3} - \log (E_0 - E_f).$$

The plot of this equation with $\log (E_t - E_f)$ as ordinate and t as abscissa is a straight line. The slope of the line is $-\frac{kc}{2.3}$ and where it cuts the ordinate is $\log (E_0 - E_f)$. The value for E_0 can be calculated from equation (2), but it can also be obtained by extrapolation of the optical density decrease to t = 0. k is a measure of the specific activity of the ribonuclease, related to the concentration of the enzyme in the assay mixture (units/mg. protein in the assay mixture). kc is the activity of the enzyme.

Kunitz defines a unit as the amount of enzyme in 1 ml. which, under the conditions of his assay (0.05% ribonucleate in 0.05 M sodium acetate buffer, pH 5.0, 25°C), decreases the optical density at 300 mμ from E_0 to E_f in one minute.

From this definition it follows that according to equation (1) the activity (units/ml. assay mixture) is:

$$(3) \qquad kc = -\frac{dE}{dt} \times \frac{1}{E_0 - E_f} \approx -\frac{\Delta E}{\Delta t} \times \frac{1}{E_0 - E_f}.$$

The assumptions for these calculations are given only approximately[15], but greater accuracy is unnecessary, because small differences in the results are not significant.

Calculation of the enzyme activity: Plot the optical densities E_t against the corresponding times t (min.). Use the approximately linear part of the curve to determine $-\frac{dE}{dt} \approx -\frac{\Delta E}{\Delta t}$. Determine E_0 by extrapolation to t = 0. Insert this value and E_f in equation (3) (see example). If the initial plot of the reaction rate is not linear, calculate some values for $\log (E_t - E_f)$ and determine kc according to equation (2) (see example).

Example

A pure ribonuclease solution contained 2.5 μg. protein per ml.

Experimental protocol:

Time after start of reaction	E_t	$E_t - E_f$	$\log (E_t - E_f)$
1 min.	0.500	0.159	$\bar{1}$ .2014
2 min.	0.490	0.149	$\bar{1}$ .1732
3 min.	0.479	0.138	$\bar{1}$ .1399
4 min.	0.468	0.127	$\bar{1}$ .1038
7 min.	0.445	0.104	$\bar{1}$ .0170
10 min.	0.430	0.089	
120 min.	0.343		
180 min.	0.341 (= E_f)		

Graphical extrapolation of the curve $E_t = f(t)$ to $t = 0$ gives $E_0 = 0.510$. From the first four minutes of the reaction

$$\frac{\Delta E}{\Delta t} = \frac{E_0 - E_4}{4} = \frac{0.510 - 0.468}{4} = \frac{0.042}{4} \; 0.0105.$$

Therefore according to equation (3):

$$kc = 0.0105 \times \frac{1}{0.510 - 0.341} = \frac{0.0105}{0.169} = 0.062 \textit{ Kunitz} \text{ units/ml. assay mixture.}$$

Since the ribonuclease preparation (2.5 μg. protein/ml.) was diluted 1:2 in the assay, the ribonuclease concentration was 0.00125 mg./ml. assay mixture and the preparation contained:

$$\frac{0.062 \text{ units/ml.}}{0.00125 \text{ mg./ml.}} = 50 \textit{ Kunitz} \text{ units/mg. protein.}$$

By calculation according to equation (2):

$$kc = 2.3 \times \frac{\Delta \log (E_t - E_f)}{\Delta t}$$

For $\Delta t = 3$ min., $\Delta \log (E_t - E_f) = 0.2014 - 0.1038 = 0.0976$.

$$kc = 2.3 \times \frac{0.0976}{3} = 0.075 \textit{ Kunitz} \text{ units/ml. assay mixture}$$

From the slope of the curve

$$\frac{kc}{2.3} = \frac{\Delta \log (E_t - E_f)}{\Delta t} = \frac{0.0976}{3} = 0.0325$$

the value for E_0 can also be calculated: for $t = 1$ min., $\log (E_t - E_f) = \bar{1}.2014$; equation (2) then reads

$$\begin{aligned} -(\bar{1}.2014) &= 0.0325 \times 1 - \log (E_0 - E_f) \\ \log (E_0 - E_f) &= 0.0325 + \bar{1}.2014 \\ \log (E_0 - E_f) &= \bar{1}.2339 \\ E_0 - E_f &= 0.171 \\ E_0 &= 0.171 + 0.341 \\ E_0 &= 0.512 \end{aligned}$$

This is in good agreement with the value for $E_0 = 0.510$ obtained by graphical extrapolation.

Sources of Error

The activity of ribonuclease is dependent on the salt content or the ionic strength[19]. Accordingly, with unknown salt concentrations the samples must be diluted considerably or dialysed. Large variations in ionic strength alter E_0 and E_f and are therefore easy to detect.
Measurements are always possible by comparison with crystalline pancreatic ribonuclease of known activity so long as $E_0 - E_f$ is not too small. If a very high salt concentration is required to prevent the precipitation of nucleoprotein, determine the ribonuclease with one of the precipitation methods already mentioned, or better still, with the following titrimetric method.

B. Titrimetric Method[18]

Principle

Ribonuclease hydrolyses cyclic 2′,3′-pyrimidine nucleotides to 3′-pyrimidine nucleotides. The acid equivalent liberated is determined by the amount of alkali required to keep the pH constant.

Optimum Conditions for Measurements

If the amount of alkali required for neutralization is taken as a measure of the activity, then the pH optimum is between 7.2 and 7.4. If correction is made for the degree of dissociation of the reaction products, then the optimum is at 7.0 for cytidine phosphate and at 7.2 for uridine phosphate[20]. The hydrolysis of cyclic nucleotides is also increased by raising the ionic strength[21]. Cyclic cytidine phosphate is hydrolysed at about twice the rate of cyclic uracil nucleotide[20].

Reagents

1. Cyclic cytidine-2′,3′-phosphate or cyclic uridine-2′,3′-phosphate
   The commercially available barium salts (*e.g.* Schwarz Bio-Research Inc.) are usually only moderately pure. Since impurities (hydrolysis products) inhibit the reaction, the purer uridine phosphate is preferable although it is hydrolysed at a considerably slower rate than cytidine phosphate.
2. Ethylene-diamine-tetra-acetic acid,
   disodium salt, EDTA-$Na_2H_2 \cdot 2\ H_2O$ (*e.g.* Komplexon III)
3. Sodium chloride, A. R.
4. Hydrochloric acid, A. R., 0.1 N
5. Sodium hydroxide, A. R., 0.1 N
6. Sodium hydroxide, A. R., 0.005 N
7. Ribonuclease, RNase
   from pancreas, crystalline; commercial preparation, see p. 997.

Preparation of Solutions

Singly distilled water is sufficient for the preparation of solutions I—III, but IV must be prepared with doubly distilled water.

I. Cyclic nucleotide (*ca.* 0.015 M):
   Dissolve 56 mg. of the barium salt in 10 ml. distilled water.

II. Ethylene-diamine-tetra-acetate (3.3×10^{-4} M EDTA):
   Dissolve 12.5 mg. EDTA-$Na_2H_2 \cdot 2\ H_2O$ in 100 ml. distilled water.

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III. Sodium chloride (0.1 M):
Dissolve 5.845 g. NaCl in distilled water and make up to 1000 ml.

IV. Ribonuclease, RNase (100 μg. protein/ml.):
Dissolve 5 mg. ribonuclease and 0.5 mg. EDTA-$Na_2H_2 \cdot 2\ H_2O$ in 50 ml. doubly distilled water.

Stability of the solutions

Prepare only a half day's requirement of solution I at a time. Prepare 0.005 N NaOH freshly each day (or titrate a new portion of the 0.1 N stock solution against HCl). Ribonuclease solutions containing EDTA (to prevent inactivation by heavy metals) are stable for about 2 months at 2 to 4° C.

Procedure

Preliminary remarks: During the hydrolysis the pH of the reaction mixture must not vary by more than 0.04 from the pH to which it was originally adjusted. The speed of stirring and the depth of immersion of the electrodes and the burette must not be altered during the assay (10—15 min.). The pH-meter should be checked with a standard buffer before and after every assay. If the pH changes by more than 0.02 in a series of measurements with an average of more than 0.01 units, then the results should be discarded. The micro-burette should be tested occasionally (at least before its first use) for leaks (fill with 0.05 N NaOH and immerse in 3 ml. of water); there must be no change in pH within 15 min. under the experimental conditions (stirring speed, immersion depth, *etc.*). The method depends more on obtaining a constant reading with repeated standardization than on the absolute accuracy of the pH-meter. The method can be adapted for automatic titration.

Enzymatic reaction

Final volume: 3 ml.; room temperature (either work in a constant temperature room or stand the small beaker, which is used as a reaction vessel, in a beaker through which is circulated liquid at a constant temperature). Stir the reaction mixture magnetically. Titrate from a micro-burette (the capillary tip is immersed in the reaction mixture) which has been calibrated. To prevent the absorption of atmospheric CO_2 direct a stream of nitrogen on to the surface of the reaction solution. Measure the pH with a direct reading pH-meter *).

Pipette successively into the reaction vessel (a very small beaker):

1.0 ml. nucleotide solution (I)
0.2 ml. EDTA solution (II)
1.0 ml. NaCl solution (III)**)
doubly distilled water, so that the volume after addition of the sample is 3.0 ml.

Adjust to pH 7.20 with 0.1 N NaOH or HCl; check the stability of the pH for several minutes. Adjust the sample to pH 7.20 (can be omitted with samples of low buffering capacity). Start the reaction by the addition of

0.1—0.8 ml. sample.

For about 10 min. add sufficient 0.005 N NaOH every 40 to 80 sec. so that the solution, which becomes acid due to the enzyme action, maintains a pH of just over 7.20. Observe the needle of the pH-meter and record the time taken for the needle to pass through pH 7.20. Plot the

*) *e.g.* Type H 2 of Beckman Instruments.
**) If necessary, take into account the ionic strength of the sample.

time (abscissa) against the amount of NaOH required (ordinate). The line does not pass through the origin. In the evaluation the first 90 sec. is disregarded, because part of the pH change on addition of the enzyme cannot be ascribed to its action on the substrate.

Calculations

During the first 15 minutes of the reaction 2 to 3% of the substrate is hydrolysed, so the concentration of inhibitory hydrolysis products is still low and therefore the initial rate of the reaction is measured. The relationship between the amount of enzyme and the rate of hydrolysis (μequiv. NaOH/min.) is therefore linear. To obtain the activity in *Kunitz* units assay a standard preparation with the spectrophotometric method at the same time.

The following definition of a unit is proposed: A unit is the amount of enzyme which hydrolyses an amount of uridine-2′,3′-phosphate equivalent to 0.1 μequiv. NaOH per minute under the conditions of the assay. Since the uridine phosphate formed is practically completely dissociated at pH 7.2, a correction for the undissociated nucleotide is unnecessary; at lower pH, for example, in the determination of heparin (p. 82), a correction is necessary.

To calculate the results plot the NaOH required (μequiv. *) against the time and draw a parallel to this line through the origin. Read off the μequiv. NaOH per minute. According to the definition of the unit this value is multiplied by 10 to give the "titrimetric units" in the reaction mixture. To calculate the number of units in the sample it is necessary to divide by the amount (ml.) of the sample taken for the assay.

Example

10 μg. ribonuclease dissolved in 0.2 ml. water was analysed. It required 0.036 μequiv. NaOH/min. $0.036 \times 10 = 0.36$ titrimetric units: therefore a unit corresponds to 27.8 μg. ribonuclease.

Conversion to other units

For crystalline ribonuclease a value of 60 *Kunitz* units (KU)/mg. was obtained (p. 795), *i.e.* 0.060 KU/μg. The same preparation contained 0.036 titrimetric units/μg. Therefore 1 titrimetric unit corresponds to 1.67 *Kunitz* units or a *Kunitz* unit is equivalent to 0.6 titrimetric units.

Sources of Error and Specificity

Apart from ribonuclease, no enzyme is so far known which can catalyse the hydrolysis of cyclic pyrimidine nucleotides. The removal of thermolabile phosphatases, which is necessary when ribonucleic acid is the subtrate for the assay of activity, can be omitted when cyclic pyrimidine nucleotides are used as substrate. Proteins interfere much less in the reaction with cyclic pyrimidine nucleotides than in that with ribonucleic acid. On the other hand the titrimetric method is considerably less sensitive. Ribonuclease can only be determined sufficiently reliably from 2 μg. per reaction mixture, while with the spectrophotometric method 0.25 μg. per reaction mixture can be determined. The precipitation methods permit a reliable measurement of up to 0.003 μg. ribonuclease[16], but do not exclude that the action of several enzymes is determined.

*) 1 μequiv. corresponds to 1 ml. 0.001 N NaOH; 1 ml. 0.005 N NaOH corresponds to 5 μequiv.