Spectrophotometric Assay of Bovine Pancreatic Ribonuclease by the use of Cytidine 2':3'-Phosphate

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Many methods have been described in the literature for determining the activity of ribonuclease (McDonald, 1955); most of these employ ribonucleic acid as substrate and are not suitable for kinetic investigations because it is not clear which step in the reaction sequence is measured. The action of the enzyme on ribonucleic acid or pyrimidine nucleotide 3'-esters involves two successive reactions: the formation of cyclic phosphates by the attack of the adjacent 2'-hydroxyl group on the phosphorus atom of those phosphate groups attached to the 3'-position of pyrimidine nucleotides; the cyclic esters are subsequently hydrolysed to give the 3'-nucleotides. Moreover, alterations in structural configuration of the ribonucleic acid are involved in the degradation and these may be included in the measure of the enzyme action, e.g. the change in ultraviolet-absorption spectrum which is the basis of the Kunitz (1946) method. Thus the only measure of the activity of ribonuclease which is well defined kinetically is its ability to catalyse the hydrolysis of pyrimidine cyclic nucleotides.

Richards (1955) has described a method for determining the hydrolysis of uridine 2':3'-phosphate which depends on differences in the absorption spectra of the reactants and products. He worked with limited amounts of substrate and was not able to investigate fully the potentialities and limitations of the method. The differences in extinction are large only in those regions of the absorption spectra where the extinction changes rapidly with wavelength, and apparent deviations from Beer's law are therefore to be expected. This difficulty can be overcome but is not always appreciated when new analytical methods are developed. Failure to take these optical factors into account would invalidate conclusions based on kinetic investigations. The method described here depends on the differences in absorption spectra of cytidine 2':3'-phosphate and cytidine 3'-phosphate and is thus similar to that of Richards (1955); the conditions have been worked out to take account of the optical factors.

MATERIALS

Cytidine 2':3'-phosphate. The potassium salt monohydrate described in the preceding paper was employed.

Cytidine 3'-phosphate. This was prepared according to the method of Harris, Orr, Roe & Thomas (1953).

2-Amino-2-hydroxymethylpropane-1:3-diol (tris). This compound (L. Light and Co. Ltd.) was dissolved in boiling methanol-water (20:3, v/v), the solution filtered and the tris allowed to crystallize. The product was collected by filtration, washed with a little methanol and recrystallized. Equivalent weight (by titration with HCl): found, 120.7; calculated, 121.1.

Tris buffer. This was prepared by dissolving 3.0285 g. of tris and 1.5725 g. of NaCl in water, adding 23.055 m-moles of HCl and diluting to 250 ml. with water. pH (glass electrode) at 25°: 7.13. $-\log[H^+]$ (calculated from the thermodynamic data of Bates & Pinching, 1949): 7.00; I , 0.2; total tris, 0.1 M.

Water. Distilled water was passed successively through columns which had previously been steamed out, containing De-Acidite FF, Zeo-Karb 225 and finally Bio-Deminolit (The Permutit Co. Ltd., London). Precautions were taken to protect the final effluent from contamination by atmospheric CO₂. The purity was checked occasionally by conductivity measurements.

Hydrochloric acid. Constant-boiling HCl was prepared according to Vogel (1951, method B); it was diluted to the concentration required.

Ribonuclease. This was lot no. 21,630 obtained from Armour and Co. Ltd., Hampden Park, Eastbourne, Sussex.

EXPERIMENTAL

Spectra of cytidine 2':3'-phosphate and cytidine 3'-phosphate. The spectrum of cytidine 2':3'-phosphate (0.25 mg. dissolved in 2.5 ml. of tris buffer) was measured against a blank of tris buffer (2.5 ml.). Enzyme solution (0.01 ml., 8 mg./ml. of tris buffer) was added to both cells; after 2 hr., when the reaction was completed, the spectrum was again read and assumed to be that of cytidine 3'-phosphate. Paper chromatography as described in the preceding paper confirmed the absence of any unhydrolysed cyclic phosphate. The spectra are shown in Fig. 1. The difference spectrum, shown in Fig. 2, was calculated, the small volume change caused by the addition of the enzyme solution being neglected.

The absorption maxima for cytidine 2':3'-phosphate and cytidine 3'-phosphate are respectively at 268 and 271 m μ ; there is an isosbestic point at 265 m μ . The maximum difference in extinction coefficients is at 286 m μ , where the ratio of the extinctions is 1.495.

Stoichiometry of reactant and product. For the determination of activity of ribonuclease it is advantageous to work at a wavelength where there is a large difference in the extinctions of the substrate and product. This necessitates working in a region of the absorption spectra where the extinctions change rapidly with wavelength. In these circumstances, apparent deviations from the Beer-Bouguer relationship are to be expected. Accordingly, the stoichiometry of both cytidine 2':3'-phosphate and cytidine 3'-phosphate have been determined at a number of wavelengths, and the maximum substrate concentrations which can be investigated with a cell of 1 cm. path length and a slit width of 0.6 mm. (Hilger Uvispek model no. H 700.307) are: at 284 m μ , 0.16 mg./ml.; at 288 m μ , 0.20 mg./ml.; at 292 m μ , 0.4 mg./ml.

Mixtures of cytidine 2':3'-phosphate and cytidine 3'-phosphate gave extinctions identical with those calculated on the assumption that the extinctions of the components are additive.

As is to be expected in regions where the extinction changes rapidly with wavelength, the measured values of the extinction increase con-

siderably as the slit width is reduced. Therefore it is important to work at a constant slit width if reproducible results are to be obtained (see Fig. 3) and, slit widths as narrow as possible should be

Fig. 2. Difference spectrum (cytidine 3'-phosphate—cytidine 2':3'-phosphate; each 0.1 mg./ml.).

Fig. 1. Absorption spectra of cytidine 2':3'-phosphate (0.1 mg./ml.) and the product obtained from it by the action of bovine pancreatic ribonuclease. Spectra were determined in tris buffer (pH 7.13; I 0.2). —, Cytidine 2':3'-phosphate; - - -, hydrolysis product.

Fig. 3. Variation of extinction with slit width of the potassium salt of cytidine 2':3'-phosphate dissolved in tris buffer (pH 7.13; I , 0.2). ●, 0.18 mg./ml., determined at 284 m μ ; ○, 0.25 mg./ml., determined at 288 m μ ; ▲, 0.70 mg./ml., determined at 292 m μ .

used so as to extend the region in which extinction is proportional to concentration.

Determination of the kinetics of hydrolysis of cytidine 2':3'-phosphate. In all instances the reaction was carried out in stoppered quartz cells in a thermostatically controlled cell holder maintained at $25 \pm 0.2^\circ$. The volume of the reaction mixture was 2.5 ml.; readings were made at $284 \text{ m}\mu$, slit width 0.6 mm.

It is important that the extinction of the reaction mixture at zero time, before any hydrolysis has taken place, should be known accurately since it is difficult to determine this by extrapolation of the progress curve. Two methods of following the reaction have been employed. Both enable the extinction at zero time to be determined.

(1) Differential method, with a blank of the same composition as the reaction mixture without the enzyme. Allowance was made for the small extinction due to the enzyme. In most instances the reaction was started by adding the substrate (0.25 ml.) to the other components of the reaction mixture, in a stoppered quartz cell. The contents were rapidly mixed by inversion; a first reading may be obtained within 20 sec. This method has the

advantage that the scale readings are lower than if the blank consisted of buffer alone, enabling greater precision to be achieved.

(2) Direct method, with a blank consisting of the reaction mixture without substrate. The reaction solution, without enzyme, was first read against a blank consisting of the same volume of buffer. Enzyme solution was added to both cells and the extinction at $284 \text{ m}\mu$ determined at suitable times. By allowing for the increase in volume on addition of the enzyme solution, the extinction of the reaction mixture at zero time can be calculated.

Examples of some progress curves are shown in Fig. 4.

Determination of initial velocities. Because of the marked curvature of the progress curves it is difficult to determine the initial velocities by drawing tangents at the origin. The following method was found satisfactory. The extinction increase corresponding to complete hydrolysis is required and was usually obtained by allowing the reaction to go to completion. For reaction mixtures with low enzyme concentrations it can be calculated.

In Fig. 5 plots of $\log(A-x)$ against time are shown, where A is the change in extinction for complete reaction and x is the change in extinction at time t . These plots are essentially linear in the early stages of the reaction. Thus $[d \log(A-x)]/dt$ can be obtained. It is possible to obtain the initial velocity in terms of extinction changes from:

$$V = \frac{dx}{dt} = -2.303A \frac{d \log(A-x)}{dt} \quad (1)$$

The linearity of the plots in Fig. 5 suggests that the reaction is first-order with respect to both enzyme and substrate, in the concentration range investigated.

$$-\frac{dS}{dt} = kES, \quad (2)$$

where S is the concentration of substrate remaining at time t and E is the enzyme concentration. Integration of equation (2) gives:

$$\ln \frac{A}{A-x} = kEt, \quad (3)$$

which is consistent with the experimental observations. The slight deviation from linearity which occurs as the reaction approaches completion is probably due to product inhibition, which is known to occur for ribonuclease (Davis & Allen, 1955).

The variation of initial velocity with enzyme concentration is shown in Fig. 6; this demonstrates the high degree of precision of the analytical technique.

Method suitable for the rapid assay of ribonuclease. Since the initial velocity of the hydrolysis of

Fig. 4. Examples of some progress curves of the hydrolysis of cytidine 2':3'-phosphate by ribonuclease in tris buffer (pH 7.13; I , 0.2). In all cases the substrate concentration was 0.1 mg./ml. Enzyme concentrations ($\mu\text{g./ml.}$): ●, 5; ○, 10; ▲, 20; △, 30; ×, 35. The broken line represents maximum value of the increase in extinction for an enzyme concentration of 0.035 mg./ml. The maximum values associated with the other enzyme concentrations differ slightly from this owing to the different values of the extinction due to the enzyme.

cytidine 2':3'-phosphate is proportional to the enzyme concentration (eqn. 1), preparations of ribonuclease can be assayed by following the progress curve under standard conditions and calculating the initial velocity. Although accurate, this is unsatisfactory if a large number of determinations is required.

If the initial substrate concentration is fixed, the reciprocal of the time required for any given fraction of the substrate to be hydrolysed, i.e. for the extinction to change by a fixed amount, should be proportional to the enzyme concentration. This will be true if the reaction is zero or first-order with respect to substrate, and first-order

with respect to the enzyme. Good straight lines are obtained up to an enzyme concentration of 35 $\mu\text{g./ml.}$

A simpler and rapid assay method which is less exact is to measure the increase in extinction in a given short reaction time, i.e. to assume that the initial part of the progress curve is straight and the slope proportional to the enzyme concentration. This is satisfactory with a substrate concentration of 0.1 mg./ml. provided that the increase in the extinction does not exceed 0.11 (up to 30% hydrolysis). Again, good straight lines are obtained up to an enzyme concentration of 35 $\mu\text{g./ml.}$ The method has been applied with success to the assay of ribonuclease fractions emerging from ion-exchange resins (C. A. Ross, unpublished work).

Fig. 5. Examples of plots used for the determination of the initial velocity of the hydrolysis of cytidine 2':3'-phosphate catalysed by ribonuclease. The text should be consulted for definition of A and x . The full lines represent the regions of linearity of the curves and take into account points which are not shown. To avoid confusion, no values before 1 min. have been plotted. The substrate concentration was 0.1 mg./ml. throughout and the enzyme concentrations ($\mu\text{g./ml.}$) were: $\bigcirc$, 5; $\bullet$, 10; $\triangle$, 20; $\blacktriangle$, 30; $\times$, 35.

DISCUSSION

Unknown concentrations of ribonuclease may thus be determined by comparison with standard samples of crystalline enzyme. Suitable conditions are: pH, approximately 7.0 and a fairly high ionic strength to minimize the effects of changes in the

Fig. 6. Plot of initial velocity against enzyme concentration for hydrolysis of cytidine 2':3'-phosphate catalysed by ribonuclease. The substrate concentration was 0.1 mg./ml.

substrate during the reaction; wavelength, 284 m μ (or within the range 284–290 m μ); initial substrate concentration, within the range 0.1–0.3 mg./ml.; slit width as narrow as possible. The final decision on slit width will depend on the conditions of the experiment and the sensitivity and stability of the spectrophotometer. The most important factor is that it should remain constant throughout a set of measurements.

SUMMARY

1. A spectrophotometric assay of bovine ribonuclease has been developed based on the change in ultraviolet-absorption spectrum resulting from the hydrolysis of cytidine 2':3'-phosphate.

2. The relevant optical and kinetic details have been carefully investigated and satisfactory conditions for the assay have been delineated.

3. Simple modifications which allow rapid assays of somewhat lower precision are also described.

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Structural Changes in Collagen

THE ACTION OF ALKALIS AND ACIDS IN THE CONVERSION OF COLLAGEN INTO EUCOLLAGEN

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The chemical and structural changes which the collagen of native adult mammalian skin, bone and tendon can undergo are difficult to follow because of the insolubility of the protein and the presence in small quantities of other substances which can be completely eliminated only by reagents which may modify the collagen. Reaction with 1-fluoro-2:4-dinitrobenzene is one of the most satisfactory methods of following the changes, since it can indicate the release of free α -amino groups. These may be formed either from the rupture of peptide bonds, from the removal of substances masking the groups or from changes of configuration which remove steric difficulties (see Steven & Tristram, 1958, for lysine ϵ -amino groups). Bowes & Moss (1953) and Grassmann & Hörmann (1953) have shown native ox-hide collagen to be without detectable *N*-terminal residues, whereas small quantities of end groups have been shown to be released by certain treatments. Bowes & Moss (1953), after using 8M-urea, found a small amount of *N*-terminal aspartic acid (1 mole/1400 kg. of collagen) and, after using alkali at pH 13.0 for 14 days, found *N*-terminal aspartic acid, glutamic acid, phenylalanine and glycine, with a residue total of 1 mole/130 kg. of collagen. Deasy (1958),

with a similar collagenous material, found *N*-terminal aspartic acid, serine and glycine in small quantities after a very mild treatment at about pH 8.6.

The conversion of collagen into gelatin of high molecular weight often requires, as an initial stage, the prior treatment of collagen in alkali or acid. The quantities of *N*-terminal residues found in gelatins by Courts (1954*a*) are substantially in excess of those given above for collagen, and Courts (1958) showed, in an extreme case, that the severe changes in protein chain length, which take place in the overall process whereby collagen is converted into gelatin, occur mainly during the initial pretreatment with cold alkali rather than as a result of hydrolyses during hot extraction of the gelatin. The present work describes in more detail the changes brought about in collagen by alkalis and acids with *N*-terminal-residue assay as the principal means of assessing changes.

Parallel with the changes in collagen during pretreatment is the solution in the alkaline and acid reagents used of substances which may play a part in the stability of the structure of native collagen. Partridge (1948) suggested that chondroitin sulphate in cartilaginous tissue acted as a multivalent