Some Factors Which Affect the Enzymatic Digestion of Ribonucleic Acid

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The results of Klee and Richards (1) are in disagreement with the conclusions of Anfinsen et al. (2–4) that concentrated urea has no effect on the activity of bovine pancreatic ribonuclease. The possibility exists that differences in enzymatic activity might be also caused by alteration of the substrate rather than of the enzyme upon treatment with these agents. In examining the effect of conditions which would be expected to influence the secondary structure of ribonucleic acid (5), we have found that concentrated urea both stimulates and inhibits the action of ribonuclease upon ribonucleic acid (depending upon the assay procedure). Some of our findings are summarized in this paper.

EXPERIMENTAL

Materials

Yeast RNA as prepared by the method of Crestfield et al. (6) is hydrolyzed more rapidly and at a more constant rate by RNase under the conditions of our assay than are commercial RNA preparations. Other advantages of this RNA are the lower oligonucleotide content of the RNase-free blanks and the absence of color or turbidity at concentrations which are sufficiently high for polariscopic observations. Solutions of RNA were prepared within 2 hours of use by allowing the freeze-dried material to dissolve in water or in 8 M urea (except where otherwise indicated) with occasional stirring. Cytidine 2',3'-cyclic phosphate was purchased as the barium salt from Schwarz Laboratories and converted to the sodium salt before use. Crystalline bovine pancreatic RNase (Lot No. 381-059) was obtained from Armour and Company. Reagent grade urea was recrystallized from ethanol.

Methods

The activity of RNase was measured by several different procedures. Activity based upon liberation of acid soluble oligonucleotides was measured with a modification of an existing method for RNase activity (7, 8). The general procedure was as follows. One ml. of 1 per cent RNA was added to 1.5 (or 2.0) ml. of RNase in buffer of suitable pH and ionic strength at 30°C. After 2 or 4 minutes, 1 ml. of 0.75 per cent uranyl acetate in 25 per cent perchloric acid (hereafter designated uranyl acetate-HClO4) was added. The mixture was cooled in an ice-water bath for 15 minutes and then centrifuged at 8°C. Aliquots (0.1 ml.) of the supernatant were promptly diluted with 3.0 ml. of water and the increased absorbancy at 260 μm over corresponding RNase-free blanks was measured. These blanks were carried through the whole experimental procedure for every determination.

RNase activity was also measured from the rate of acid formation at constant pH, with the use of a Radiometer TTT-1 pH-Stat to control the addition of 0.01 M NaOH from a motor driven syringe burette to a vessel which contained substrate and other additions as indicated for each experiment. This vessel was stirred magnetically, jacketed with circulating water at constant temperature, and protected from atmospheric carbon dioxide by a gentle stream of nitrogen gas. RNase was added manually from a second syringe burette after the substrate solution reached temperature and pH equilibrium.

The activity of RNase as measured by the increase in extinction of RNA was observed in a Cary model 14 Spectrophotometer, with identical solutions of RNA and buffer in matched 1-cm. cuvettes in each beam. To initiate the reaction, RNase was added by micropipette to the sample cell and the absorbancy difference between the two beams at 258 μm was recorded graphically.

The change in optical rotation of RNA as a result of RNase activity was measured in a Zeiss-Winkel polarimeter with the use of the D line of sodium and a 2-dm. tube containing substrate, enzyme, or other additions at 25°C.

RESULTS

Influence of Ionic Strength and pH on RNase Activity—As is now well established, the activity of RNase is dependent upon the presence of nonspecific electrolytes. The ionic requirement was found to vary with pH, as is illustrated in Fig. 1. At pH 7.3, the ionic strength optimum was about 0.06 to 0.10, as determined both by liberation of acid-soluble oligonucleotides and of acid. At pH 5.2 however, the ionic strength optimum was about 0.20, as judged by oligonucleotide measurements only (inasmuch as formation of secondary phosphoric acid groups cannot be measured directly at this pH).

The optimas for pH and ionic strength were interdependent. As the ionic strength was increased, the pH optimum for the liberation of acid-soluble oligonucleotides by RNase was found to shift toward a lower pH, as shown in Fig. 2. In addition to these effects, the ionic environment also influenced the course of hydrolysis of RNA. The initial rate of acid formation from RNA by RNase at pH 7.2 was virtually the same at low and high ionic strengths (Fig. 3), but as the reaction proceeded, the rate became appreciably slower at low salt concentrations.

Effect of Urea upon RNase Activity—The liberation of acid-
soluble oligonucleotides from RNA by the action of RNase was markedly increased by the presence of increasing concentrations of urea (Fig. 4). This urea effect was more marked at pH 5 than at pH 7 (Figs. 4 and 5). A brief but consistent lag of about a half minute was repeatedly observed after mixing RNase with RNA, both at pH 5 and pH 7. In the presence of urea, the delay was somewhat shorter (Fig. 5). Under almost exactly the same conditions, however, there was no lag in the production of acid (Fig. 3). Pretreatment of either RNA or RNase had no effect on the liberation of acid-soluble oligonucleotides over that found when RNA, RNase, buffer and urea were simultaneously mixed, (Table I). An appreciable increase was observed even when urea was added just before or after the RNase activity was stopped with uranyl acetate-HClO₄ reagent. This urea effect could be completely reversed by removal of the urea by dialysis before adding RNase (Table II). The blank values from RNA were also slightly increased by addition of urea.

Attempts were made to further investigate the effect of urea on the solubility of the products of the reaction, in neutral or slightly acid media. Unfortunately, the enzymatic action of

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We have had similar experiences with commercial RNA (Nutritional Biochemicals Corporation, Cleveland, Ohio) as substrate.
FIG. 5. The rate of oligonucleotide liberation after addition of 1 ml. of 1 per cent RNA in water (Δ and ○) or 8 M urea (● and △) to 6 μg. of RNase in 2 ml. of 0.1 M acetate, pH 5.2 (○ and ●) or to 0.6 μg. of RNase in 2 ml. of 0.1 M phosphate pH 7.0 (Δ and △) at 30°. The vertical axis is the increased absorbancy over the RNase-free blanks of a 1:30 dilution of the supernatant after addition of 1 ml. of uranyl acetate-HClO₄ reagent. The blank values subtracted ranged from 0.07 (in the absence of urea) to 0.15 (in the presence of urea).

TABLE I
Acid-soluble oligonucleotides liberated from ribonucleic acid by RNase as affected by conditions of urea treatment

Experimental conditions: To 1 ml. of 0.1 M acetate buffer, pH 5.0, with 5 μg. of RNase and 1 ml. of water or 8 M urea was added 1 ml. of 1 per cent RNA in water or 8 M urea. After 3.75 minutes at 30°, 1 ml. of water, 2.6 M urea, or 10.4 M urea was added and at 4.0 minutes, 1 ml. of uranyl acetate-HClO₄ reagent was added. After centrifuging at 3°, the supernatant was diluted 1:30 and the increased absorbancy over corresponding RNase-free blanks was measured at 260 μm.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urea concentration M</th>
<th>Absorbancy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No urea</td>
<td>0</td>
<td>0.06</td>
</tr>
<tr>
<td>RNA pretreated with 8 M urea, 3 hours</td>
<td>2.6</td>
<td>0.37</td>
</tr>
<tr>
<td>RNase pretreated with 4 M urea, 15 minutes</td>
<td>2.6</td>
<td>0.35</td>
</tr>
<tr>
<td>Urea added at start of reaction</td>
<td>2.6</td>
<td>0.36</td>
</tr>
<tr>
<td>Urea added 15 seconds before addition of uranyl acetate-HClO₄</td>
<td>2.6</td>
<td>0.15</td>
</tr>
<tr>
<td>Urea added after addition of uranyl acetate-HClO₄</td>
<td>2.6</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* In every case, controls of RNA plus urea, but without enzyme, were run through the incubation and precipitation treatment. These absorbancy values (0.03 for the first control, with no urea, and 0.04 for each of the other controls) were subtracted from the experimental values obtained with the enzyme.

Fig. 6. The change in specific rotation during the enzymatic hydrolysis of RNA in the presence (Δ—Δ) and absence (○—○) of 3.2 M urea. The rotation of a solution containing 100 mg. of RNA and 10 μg. of RNase in 25 ml. of 0.03 M phosphate, pH 7.3 (with or without urea), was observed at 25° in a 2-dm. tube.
RNase on RNA, under the conditions of high substrate concentrations in these experiments, could not be stopped by $10^{-5}$ or $10^{-4}$ m CuSO$_4$, $10^{-4}$ m AgNO$_3$, $10^{-4}$ m polyxenylphosphate, or 0.025 per cent heparin.

The augmented yield of acid-soluble oligonucleotides observed in the presence of urea probably does not represent a true stimulation of RNase activity. Acid liberation from both RNA and cytidine cyclic phosphate by RNase was inhibited by concentrations of urea which stimulate oligonucleotide formation (Table III). The degree of inhibition was much less with RNA than with the cyclic nucleotide. Moreover, the rate of breakdown of RNA by RNase as measured by two other methods did not appear to be stimulated by urea. On enzymatic hydrolysis of RNA, its specific rotation decreases and its extinction increases. In neither case was this enzymatic hydrolysis stimulated by urea (Figs. 6 and 7). If these changes in optical properties of substrate may be taken to represent the first step in the enzymatic attack while the liberation of acid represents the second step, it would appear that neither action of RNase is stimulated by urea.

Despite the similar effects of electrolytes and urea on some properties of RNA, urea could not substitute for the electrolyte requirement of RNase.

**DISCUSSION**

The lag in the appearance of oligonucleotides is probably an artifact of the precipitation procedure because no similar delay is found when the course of RNA hydrolysis is followed by acid formation or by measurements of absorbance or optical rotation. In the initial attack by RNase, the first oligonucleotide fragments created are probably too large or too firmly hydrogen-bonded to neighboring chains to escape precipitation by the uranyl acetate-HClO$_4$ reagent. Precipitability of RNA by this reagent appears also to be markedly temperature dependent. Some degradation of RNA by perchloric acid has been previously observed (9). We have observed that the amount of acid-soluble oligonucleotides is less if the precipitate is centrifuged in the cold than if centrifuged at room temperature.

The hydrolysis of RNA by RNase has been repeatedly shown to depend upon the presence of nonspecific electrolytes (10-13). This dependence may be in part a reflection of the profound influence of dilute salt upon the cooperative structure of the substrate as revealed by changes in its viscosity, optical properties, and degree of ionization. It may therefore not be coincidental that the ionic strength optimum of RNase activity toward RNA at pH 7 is about the same electrolyte concentration which transforms RNA to a highly oriented form (5). In addition to the effects of ionic environment on RNA structure, nonspecific electrolytes appear to be required for enzymatic activity of RNase itself. There is an ionic requirement (14, 15) for the hydrolysis by RNase of nucleoside 2',3'-cyclic phosphates. This type of substrate presumably would be unaffected by the presence of electrolytes. However Buzzell and Tanford (16) have shown that the compact structure of the RNase molecule is unaffected by wide variations in the ionic strength. There appears to be yet another effect of the ionic concentration upon the enzyme reaction. In contrast to our earlier experiences with cytidine cyclic phosphate hydrolysis (14), higher concentrations of KCl maintained the liberation of acid from RNA at a more constant rate, suggesting that electrolytes may minimize the inhibition of RNase caused by accumulation of hydrolysis products.

The influence of the pH of the medium upon the ionic strength optimum for acid liberation from RNA by RNase has previously been reported by Edelhoch and Coleman (13). This shift of the pH optimum in the acid direction in the presence of higher concentrations of electrolytes could be caused by changes in the ionization of either the enzyme or the substrate, thereby altering their coulombic interaction.

A simple explanation of the effect of urea on RNase activity in these experiments would be that urea disrupts the secondary or hydrogen-bonded structure of RNA and of the products of the enzymatic reaction. Since some increase of acid-soluble oligonucleotides is obtained when partially digested RNA is treated with urea (either just before or after the enzymatic reaction is stopped with uranyl acetate-HClO$_4$ reagent), it appears that urea does increase the solubility of the oligonucleotides in the presence of uranyl acetate-HClO$_4$ to some extent. By disrupting the secondary structure of the substrate, RNA, the urea would therefore increase the effective substrate concentration due to the greater number of hydrolyzable groups which are presented to the enzyme. Such an occurrence could explain the somewhat lesser inhibition by urea of the formation of acid from RNA than from the cyclic phosphate by RNase, and the marked increase of oligonucleotide formation in the presence of initial high concentrations of urea. Thus it would appear that urea exerts a reversible denaturing influence upon RNA, rendering it more susceptible to enzymatic digestion, in a manner reminiscent of the effect of denaturation upon enzymatic digestion of proteins (17).

**SUMMARY**

1. The ionic requirement for maximal ribonuclease activity varies with pH, the optimal ionic strengths being 0.06 to 0.1 at pH 7.3 and 0.2 at pH 5. The optima for pH and ionic strength are interdependent, the pH optimum shifting toward a lower pH as the ionic strength is increased.

2. The rate of hydrolysis of yeast ribonuclease measured by the change in its specific rotation caused by ribonuclease is not stimulated by the presence of urea. The rate of hydrolysis based upon the change in extinction due to the change in hypochromicity is somewhat inhibited by urea. Urea inhibits acid liberation from yeast ribonuclease and cytidine 2',3'-phosphate by ribonuclease, but the degree of inhibition is much less with the former.
3. The formation of acid-soluble oligonucleotides is increased when ribonuclease acts on ribonucleic acid in the presence of urea. Some increase is also observed even when the urea is added just before or after the action is stopped. The urea effect can be completely reversed by removal of the urea by dialysis. It is concluded that urea acts by disrupting the secondary structure of both the nucleic acid and the products of the reaction.

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