EFFECTS OF IONIC STRENGTH ON RIBONUCLEIC ACID STRUCTURE AND RIBONUCLEASE ACTIVITY*

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Beef pancreas ribonuclease activity is markedly accelerated by increased ionic strength (1–4). Since certain physical properties of nucleic acids are known to be altered by this factor, it becomes of interest to determine whether salt concentration affects primarily the enzyme, the substrate, or both. A second factor of importance in ribonuclease (RNase) assays is that of substrate concentration. A suggestion of the influence that substrate concentration may exert on the phosphodiesterase activity of RNase can be gained from a consideration of the sensitivities of three published assays (4–6). As the data of Table I demonstrate, there is an inverse relationship between ribonucleic acid concentration and sensitivity when beef pancreas ribonuclease is assayed at pH 5.0. A quantitative investigation of the effects of ionic strength and ribonucleic acid (RNA) concentration on RNase activity, as determined by two different types of procedure, has been carried out. One assay is based on the determination of the hydrolytic products of the reaction (phosphodiesterase activity) (4) and the other on the decrease in absorbance at 300 nm ($A_{300}$) of an RNA solution as a function of time (spectrophotometric procedure) (7). The results indicate that both factors are capable of exerting profound effects on ribonuclease measurements with either type of assay.

Differences in the effects of ionic strength and substrate concentration on the spectrophotometric and the phosphodiesterase assays suggested that the reaction being measured was different in the two assays. This hypothesis was strengthened by the observation that even in the absence of RNase the $A_{300}$ of ribonucleic acid solutions was immediately decreased when the ionic strength of the solution was increased. This effect suggests that some type of non-hydrolytic alteration has occurred. Further work will be necessary to determine whether the decrease in absorbance at 300 nm caused by ribonuclease or by increased ionic strength is due to the same structural change. Vandendriessche (8) observed that the addition of RNase to a solution of RNA caused a temporary increase in the volume

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of the solution. Since a hydrolytic reaction would be expected to decrease the volume, he interpreted this effect as an alteration in the superstructure of RNA. Ribonuclease is thus similar to trypsin (9) in that it alters its substrate as a preliminary to the hydrolytic reactions.

**Materials and Methods**

Crystalline ribonuclease was obtained from Armour and Company. It was dissolved and diluted in appropriate solutions, all of which contained 0.001 per cent gelatin. Ribonucleic acid was purchased from the Schwarz Laboratories, Inc., and purified by the procedure of Vischer and Chargaff (10). The pH of all solutions was determined by means of a glass electrode pH meter, and absorbancies were determined with a Beckman DU spectrophotometer.

**Table I**

*Sensitivities of Three Ribonuclease Assays at pH 5.0*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>RNA Ionic strength</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anfinsen et al. (5)</td>
<td>3.2 0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>Dickman et al. (4)</td>
<td>1.5 0.1</td>
<td>0.91</td>
</tr>
<tr>
<td>Schneider and Hogeboom (6)</td>
<td>0.2 0.06</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Ribonuclease activity was measured by the procedure of Dickman et al. (4) at pH 5.0 or by a modification which involved the measurement of absorbance at 290 m\textsuperscript{a} directly on the supernatant solution.\footnote{We wish to thank Dr. Alan Mehler for this suggestion.} The absorbance values listed are all corrected for the absorbance of the corresponding control solution. The spectrophotometric assay of Kunitz (7) was also employed and was followed without modification. For the comparison of phosphodiesterase assay methods (Table I), the procedures of Dickman et al. (4), Anfinsen et al. (5), and Schneider and Hogeboom (6) were followed exactly, except that the incubations were all carried out for 10 minutes at 37\textdegree. The rate study (Fig. 4) was conducted at pH 5.0 at 20\textdegree at an ionic strength of 0.05. The total volume was 4.0 ml for the spectrophotometric procedure and 1.0 ml for the phosphodiesterase. The final concentration of both RNA and RNase was the same in both procedures. Ionic strength was varied by use of acetate buffer, pH 5.0.


Results

Factors Affecting Phosphodiesterase Activity of Ribonuclease

Effect of Ionic Strength on Substrate Inhibition—When ribonucleic acid concentration was varied from 0.05 to 1.8 mg. per ml. in the assay tube, and ionic strength was varied from 0.1 to 0.6, the family of curves shown in Fig. 1 was obtained. At ionic strengths below 0.6, substrate inhibition became evident at ribonucleic acid concentrations greater than 0.4 mg. per ml. This inhibition was most marked at 0.1 and decreased gradually as ionic strength was increased. At 0.6 ionic strength no substrate inhibition was detected up to an RNA concentration of 1.8 mg. per ml.

Effect of Ionic Strength on Ribonuclease Activity—When ionic strength was varied at three different RNA concentrations, the curves of Fig. 2 were obtained. In all instances a marked accelerating effect of ionic strength was noted. This effect increased as substrate concentration was increased up to the range where other factors became limiting.

The data presented thus far indicate that the ribonuclease assay of
Dickman et al. is carried out under conditions of substrate inhibition. Accordingly, assays were made at 0.5 mg. per ml. of RNA concentrations at ionic strengths of 0.3 and 0.6. As shown in Fig. 3, both curves possess steeper slopes than the curve run under the usual assay conditions but the range of ribonuclease concentrations in the straight line portion of the curves is considerably reduced. Increase in ionic strength increases the sensitivity of the assay but reduces its range.

The original assay of Dickman et al. involves dilution of an aliquot of the supernatant solution with H₂O and determination of the absorbance at 260 mμ (A₂₆₀). The absorbance of the supernatant solution can be determined at 290 mμ directly, thus avoiding the dilution step. As shown in Fig. 3, a quantitative correlation exists between the absorbance readings at the two wave lengths. Consequently, determinations were routinely carried out at 290 mμ for many of the studies here reported.

Factors Affecting Spectrophotometric Assay of Ribonuclease

Ionic Strength—When ionic strength was varied in the spectrophotometric assay, the results listed in Table II were obtained. While there was an appreciable acceleration between 0 and 0.05, at ionic strengths above 0.1 an inhibition was observed. This profound difference in the
effect of ionic strength on the phosphodiesterase and spectrophotometric assays clearly indicates that the reactions being measured in the two assays are quite different.

![Graph showing Ribonuclease assays at pH 5.0.](image)

**Table II**

<table>
<thead>
<tr>
<th>Ionic strength</th>
<th>Decrease in A$_{300}$ between 1 and 5 min. after mixing</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>0.05</td>
<td>0.070</td>
</tr>
<tr>
<td>0.10</td>
<td>0.070</td>
</tr>
<tr>
<td>0.20</td>
<td>0.062</td>
</tr>
<tr>
<td>0.30</td>
<td>0.054</td>
</tr>
<tr>
<td>0.55</td>
<td>0.037</td>
</tr>
</tbody>
</table>

To determine whether the salt concentration affects primarily the enzyme or the substrate, the influence of ionic strength on the absorptivity of RNA at 300 μμ (A$_{300}$) was studied. The data of Table III demonstrate that increased ionic strength depresses A$_{300}$ of RNA at pH 5.0. These
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results explain the apparent decreased activity of ribonuclease in the spectrophotometric assay at the higher ionic strengths since the additional salt produces the same over-all effect as the enzyme. Kunitz (7) found that RNase action caused a very small increase in the absorptivity of an RNA

**Table III**

*Effect of Ionic Strength on Absorptivity of Ribonucleic Acid*

<table>
<thead>
<tr>
<th>Added salt</th>
<th>Ionic strength</th>
<th>A$_{260}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0.920</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.125</td>
<td>0.620</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.250</td>
<td>0.600</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.375</td>
<td>0.530</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.500</td>
<td>0.530</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.00</td>
<td>0.530</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.250</td>
<td>0.465</td>
</tr>
<tr>
<td>KCl</td>
<td>0.250</td>
<td>0.545</td>
</tr>
<tr>
<td>NaF</td>
<td>0.250</td>
<td>0.600</td>
</tr>
</tbody>
</table>

**Table IV**

*Effect of Ribonucleic Acid Concentration on Spectrophotometric Assay of Ribonuclease*

Purified RNA, 8 mg. per ml., pH 5.0, was diluted with H$_2$O, then mixed with 1.1 ml. of a solution containing RNase, 4 $\gamma$ per ml., and 0.1 ml. of 2.0 M acetate, pH 5.0. Total volume, 4.0 ml.; final ionic strength, 0.05.

<table>
<thead>
<tr>
<th>RNA concentration</th>
<th>Decrease in A$_{260}$ between 1 and 5 min. after mixing</th>
<th>RNA concentration</th>
<th>Decrease in A$_{260}$ between 1 and 5 min. after mixing</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg. per ml.</td>
<td></td>
<td>mg. per ml.</td>
<td></td>
</tr>
<tr>
<td>0.075</td>
<td>0.028</td>
<td>0.500</td>
<td>0.060</td>
</tr>
<tr>
<td>0.100</td>
<td>0.033</td>
<td>0.600</td>
<td>0.068</td>
</tr>
<tr>
<td>0.200</td>
<td>0.043</td>
<td>0.800</td>
<td>0.070</td>
</tr>
<tr>
<td>0.250</td>
<td>0.052</td>
<td>1.00</td>
<td>0.100</td>
</tr>
<tr>
<td>0.400</td>
<td>0.058</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

solution at A$_{260}$. Similarly, an ionic strength of 0.25 produced by either acetate or NaCl did not alter the absorptivity of RNA at 260 m.$\mu$.

It is also noteworthy that this effect of ionic strength on A$_{260}$ is reversible. Concentrated solutions of RNA at an ionic strength of 0.25 were diluted ten times or 100 times with either H$_2$O, 0.25 M acetate, or 0.25 M NaCl. The absorptivity of the solutions diluted with H$_2$O approached those of RNA originally dissolved and diluted in H$_2$O, whereas dilution
with the salt solutions resulted in significantly lower values of $A_{300}$. All of these solutions were adjusted to pH 5.0.

**Substrate Concentration**—RNA concentration was varied under the conditions of the spectrophotometric assay of RNase. Ionic strength and enzyme concentration were held constant. As demonstrated by the data of Table IV, an increased concentration of RNA resulted in a gradual in-

![Fig. 4. Comparison of rates of ribonuclease catalyzed reactions. Curve I, spectrophotometric; Curve II, phosphodiesterase. Each tube contained purified yeast RNA, 0.5 mg. per ml., pH 5.0, RNase, 0.08 $\gamma$ per ml., and 0.05 M acetate buffer, pH 5.0. The final volume was 4.0 ml. in the spectrophotometric and 1.0 ml. in the phosphodiesterase assay. In the phosphodiesterase assay, 3 ml. of tert-butanol-acetic acid were added at the time indicated and the tube was centrifuged. 1 ml. of the clear supernatant solution was diluted with 5.0 ml. of H$_2$O and the $A_{260}$ determined. Each point represents the average of four separate determinations.](http://www.jbc.org/)

crease in the rate of change of the absorbance of RNA solutions at 300 m$\mu$. These results, typical of those obtained with many enzymes, are in marked contrast to those included in Fig. 1, Curve I.

**Comparison of Rates of Spectrophotometric and Phosphodiesterase Assays**

The data presented thus far suggest that the spectrophotometric and phosphodiesterase procedures possibly measure different reactions. Evidence that these are truly independent reactions is included in Fig. 4. The rate of the phosphodiester hydrolysis reaction was zero during the first 2 minutes. The slope increased in the interval 2 to 5 minutes, but the re-
action did not attain its maximal rate until the final 5 minutes. The rate of the reaction determined by the spectrophotometric procedure, however, remained constant in the interval 1 to 5 minutes, as first reported by Kunitz (7). These data furnish evidence that the spectrophotometric procedure measures a non-hydrolytic reaction which precedes the hydrolysis of the phosphodiester linkage. The fact that the rate of this reaction remained constant while that of the hydrolytic reaction increased indicates that these two procedures are indeed measuring independent reactions.

**DISCUSSION**

Ribonuclease action, increase in ionic strength, or increase in pH (Kunitz (7), (Fig. 3)) all decrease the absorptivity of RNA at 300 μm. On the other hand the absorptivity at 260 μm remains essentially constant during a short (5 to 10 minutes) incubation with RNase or with an increase in ionic strength. The relative constancy of the 260 μm absorbance readings suggests that no significant hydrolysis of phosphodiester linkages has occurred and supports the finding of Kunitz (7) that the rate of formation of free acid is considerably slower than the rate of the spectral shift. This interpretation is also in accord with the observations of Magasanik and Chargaff (11), who found an appreciable increase in absorbance at 260 μm with hydrolysis of RNA by alkali. It also becomes probable that this initial reaction between ribonuclease and ribonucleic acid is responsible for both the spectral shifts as measured by the spectrophotometric assay and the dilatation results of Vandendriessche (8). This author interpreted the increase in dilatation as an effect on the "superstructure" of RNA, analogous to a denaturing action. The observations that increase in ionic strength alone produces similar spectral shifts and that these are reversible, in conjunction with the fact that salt prevents denaturation of deoxyribonucleic acid (12), suggest that considerable caution should be observed in considering these effects as a denaturation of RNA. As will be discussed more fully below, it is possible that both the spectral shifts at 300 μm and the volume changes are due to increased dissociation of protons from RNA.

Although the accelerating effect of ionic strength on a number of enzymes is well known, as is the phenomenon of substrate inhibition (13), the interrelationship of these two factors with the pH optimum of an enzyme seems not to have been previously studied. In this section a preliminary explanation of the observations will be attempted. Kunitz first reported (14) that the pH optimum of RNase was in the region, pH 7.5 to 7.7. This fact has been confirmed by others with a phosphodiesterase type assay (15, 16). When the pH of RNA solutions is increased, each acidic group with a pK in that pH region dissociates to a greater extent.

Edelhoch and Coleman (3) observed that addition of neutral salt to un-
buffered solutions of RNA decreased the pH. This observation had also been made independently in this laboratory and indicates a greater dissociation of protons from acidic groups. Thus an increase in either pH or ionic strength produces an increase in the dissociation of certain acidic groups in RNA. If one assumes that the increased phosphodiesterase activity of RNase at higher pH is causally related to this greater degree of dissociation in the RNA, then it would follow that other factors which also increase dissociation (such as ionic strength) would exert a similar effect. It is noteworthy that substrate inhibition of RNase was not observed at pH 7.5 (3) or at pH 5.0 at an ionic strength of 0.6 (Fig. 1, Curve IV). Thus substrate inhibition of RNase phosphodiesterase activity would be caused by relatively undissociated RNA being bound to the enzyme.

Crystalline beef pancreas ribonuclease is capable of at least three types of action on RNA or its degradation products. These may be considered as a series of consecutive reactions: (1) non-hydrolytic alterations in structure. Such processes can be readily demonstrated at a low ionic strength (μ = 0.05) and at pH 5.0. (2) Hydrolysis of specific phosphodiester linkages to form nucleoside cyclic phosphates (17). (3) Hydrolysis of specific nucleoside cyclic phosphates to form nucleotides (17, 18). Fig. 4 demonstrates that Reaction 1 precedes Reaction 2 by a short interval. Markham and Smith have shown that Reaction 2 precedes and is faster than Reaction 3 (18).

**SUMMARY**

1. Crystalline beef pancreas ribonuclease is inhibited in its phosphodiesterase activity by excess ribonucleic acid at pH 5.0. This inhibition can be decreased or prevented by an increase in ionic strength of the medium. Substrate inhibition did not occur with the Kunitz spectrophotometric ribonuclease assay.

2. An increase in ionic strength from 0.1 to 0.6 markedly increases the phosphodiesterase activity of ribonuclease, but an ionic strength greater than 0.1 depresses the apparent catalytic effect of the enzyme in the spectrophotometric assay.

3. Increased ionic strength depresses the absorptivity at 300 mμ of ribonucleic acid at pH 5.0. This spectral effect is reversible.

4. It is concluded that the Kunitz spectrophotometric assay measures a non-hydrolytic alteration in the structure of ribonucleic acid. This alteration precedes measurable hydrolysis of phosphodiester linkages by a short time interval.

**BIBLIOGRAPHY**

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