# PANCREATIC RIBONUCLEASE-POLY G COMPLEXES: COMPLETE INHIBITION OF POLY U HYDROLYSIS

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ABSTRACT Pancreatic ribonuclease forms large complexes with poly G in 0.1 M acetate buffer solutions (pH 5.4). These are largest when the ratio, of ribonuclease to poly G concentration, is slightly less than 2. Under the same conditions lysozyme forms still larger complexes with poly U, and these are largest when the ratio, of lysozyme to poly U concentration, is about 2.5. The ribonuclease in ribonuclease-poly G complexes digests poly U. Free ribonuclease digests the poly U in lysozyme-poly U complexes. However, when the poly G concentration is about an order of magnitude greater than that required to bind all the ribonuclease, lysozyme-bound poly U is not hydrolyzed.

## INTRODUCTION

Natural protein-nucleic acid complexes (ribosomes, viruses, etc.) have particle weights that fall roughly between $10^6$ and $10^9$. They are made up of proteinaceous and nucleic acid sub-units having particle weights greater than about $10^4$; i.e., polymers already classified as macromolecules interact to form entities that are often several orders of magnitude more massive. Evidence shows this to be anything but a superficial and haphazard aggregation.

Recently (1), ribosomes were broken down into their proteinaceous and nucleic acid components. The latter were selectively recombined to form particles that were active in cell-free protein synthesis, only if they contained the right proteins and nucleic acids. Protein synthesis is a vital process. On the other hand, the intracellular breakdown and correct reconstitution of viruses involve a pathological series of processes. Thus, large protein-nucleic acid complexes can be either vital or disastrous factors at the cellular level.

The natural formation of such complexes is difficult to follow by most physical techniques, because it occurs in a cellular environment, almost hopelessly encumbered by factors beyond experimental control. An in vitro system is desirable, with well characterized proteins and nucleic acids interacting to form the super particles. One such system involving TMV protein and RNA is under critical study (2). Another has been described in this journal (3).

Some basic proteins (lysozyme, trypsin, chymotrypsinogen, pancreatic ribonuclease, etc.) interact with polyribonucleotides in weakly acidic solutions to form complexes that are especially large when [protein]/[polyribonucleotide] assumes some critical value. In some cases the light-scattering power of the mixtures is so high that this ratio is easily determined at protein and polynucleotide concentrations of the order of 10 $\mu$g/ml. Poly U-lysozyme mixtures are by far the most turbid if the right conditions are reached. A 0.1 M acetate buffer solution (pH 5.4) containing only 15 $\mu$g of lysozyme and 6 $\mu$g of poly U per ml appears opalescent in ordinary room light (see Fig. 3 of reference 3). This is remarkable because solutions containing 21 $\mu$g of either component per ml are essentially no more turbid than the buffer itself. The experiments described below were designed to compare the vulnerability to digestion by pancreatic ribonuclease, of this lysozyme-bound poly U, with that of free poly U. Both proteins form large stable complexes with poly G (not digested by pancreatic ribonuclease). The results showed that (*a*) the poly U in lysozyme-poly U complexes is completely digested by free ribonuclease, (*b*) unbound poly U is digested by the ribonuclease in ribonuclease-poly G complexes, but (*c*) poly U in lysozyme-poly U complexes is completely immune to attack by pancreatic ribonuclease if considerably more poly G is present than that amount required to bind all the ribonuclease. In the latter case, hydrolysis products that should have appeared within a few seconds did not appear within 2 hr. This introduces the interesting possibility that selective complexing of intracellular proteinaceous and nucleic acid materials may be a switching mechanism, turning on (or off) certain reactions at critical times.

## MATERIALS AND METHODS

Specifications, suppliers and notations used for all compounds are listed in Table I.

### *Light-Scattering Measurements*

These were made with a Brice-Phoenix light-scattering photometer (Phoenix Precision Instrument Company, Philadelphia, Pa.) as previously described (3). The 4358-A Hg line was

TABLE I
MATERIALS

| Compound | Specifications and supplier |
|---|---|
| Polyguanylic acid (poly G) | Sodium salt, control numbers 5272 and 11-06-314 from Miles Laboratories Inc., Elkhart, Indiana. |
| Polyuridylic acid (poly U) | Ammonium salt, control numbers 41855 and 45858 from Miles Laboratories Inc. |
| Polyadenylic acid (poly A) | Potassium salt, control number 110638 from Miles Laboratories Inc. |
| Ribonuclease | Pancreatic, 5× crystallized, lot 59616 from General Biochemicals, Chagrin Falls, Ohio. |
| Lysozyme | 3× crystallized, lot 50433, from General Biochemicals. |

used throughout. Scattered-light intensities were obtained at 45°, 75°, 105°, and 135° with the transmitted light beam. However, only the data taken at 45° and 135° are necessary here. The ordinate (G) on the figures is numerically equal to about $2.5 \times 10^5$ $R$, in which $R$ is the Rayleigh ratio (4). Other details are given in the descriptions of the experiments.

*Measurement of Hydrolysis Products*

These data were obtained in much the same way as previously described (5). However some procedural changes were necessary. As before, hydrolysis was stopped by adding 25% perchloric acid solution containing 1% (by weight) uranyl acetate, the mixture was spun at about 3000 g and the absorbance of hydrolysis products was determined at 2600 A. When the concentration of poly U hydrolysis products was quite low (as was necessarily so in experiments involving lysozyme-poly U complexes) the supernatant fluid in the centrifuge tubes was decanted directly into the Beckman (Beckman Instruments, Inc., Palo Alto, Calif.) absorption cells, without first diluting with distilled water. This increased the magnitude of errors, but kept $OD_{2600A}$ up in a readable range. This was determined relative to blanks containing the appropriate amount of poly G.

## RESULTS AND DISCUSSION

*Ribonuclease-Polypurine Complexes*

3 ml of buffer containing 60 μg of pancreatic ribonuclease per ml was added to 9 ml of buffer containing various weights of poly G per ml (0.1 M acetate buffer (pH 5.4) was used in every experiment described in this report). Results are shown in Fig. 1 *a*. After ribonuclease addition the mixtures contained 15 μg of ribonuclease and the designated weights of poly G per ml. The scattered-light intensity, at both angles, is essentially constant within about 10 min after mixing, indicating that both the number and the size of scattering centers are constant. This experiment is completely analogous to that involving the addition of lysozyme to poly C solutions (see Fig. 1 of reference 3). The important point is that the complexes are stable.

Similar experiments were carried out with poly A substituted for the poly G. [Ribonuclease] had to be increased to 30 μg/ml in order to obtain scattered-light intensities comparable to those in Fig. 1 *a*. Results are shown in Fig. 1 *b*. Both the dissymmetry ($G_{45}/G_{135}$) and the intensity vary greatly with time. In all cases the ight-scattering power of the mixtures eventually falls to insignificant values. Under these conditions there is a slow depolymerization of poly A by pancreatic ribonuclease (5). Poly G is the only homopolymer that forms stable complexes with pancreatic ribonuclease.

Experiments like those shown in Fig. 1 *a* were carried out at other fixed ribonuclease concentrations. Results are shown in Fig. 2. Scattered-light intensities, 15 min after ribonuclease addition, are plotted as a function of poly G concentration. These experiments are completely analogous to those involving lysozyme and poly C shown in Fig. 2 of reference 3. The concentration ratio at maximum intensity and dissymmetry of scattering appears to be slightly less than 2, in favor of ribonuclease.

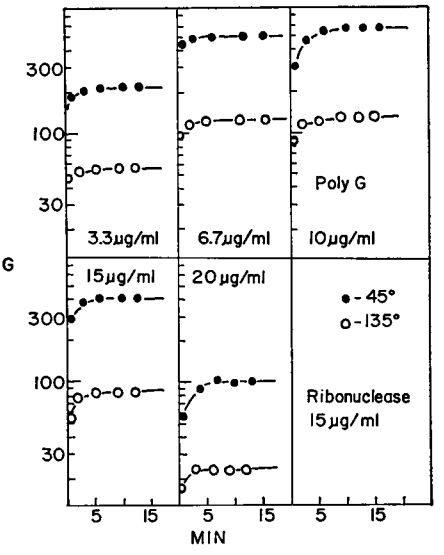


FIGURE 1 *a* Curves of scattered-light intensity vs. time, obtained by adding ribonuclease to solutions containing various amounts of poly G. Final concentrations are shown on the figure. The ordinate (G) on this and following figures is numerically equal to about $2.5 \times 10^5 R$ in which $R$ is the Rayleigh ratio. Before ribonuclease addition $G_{45}$ was never greater than about 14 at these concentrations.

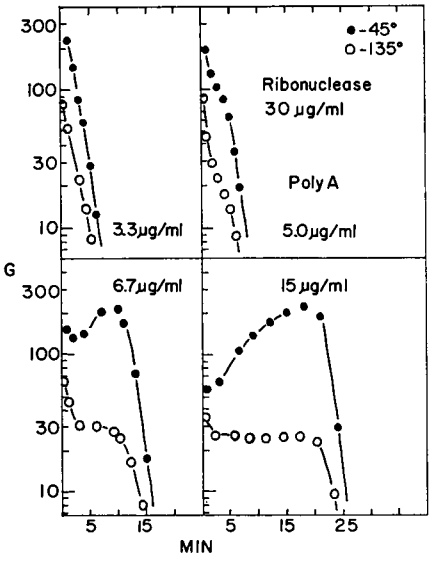


FIGURE 1 *b* Like Fig. 1 *a*, only with double the ribonuclease concentration and poly A substituted for poly G. Compare the time variation in scattering with that in Fig. 1 *a*. This is why we used poly G in the hydrolysis experiments.

At all three protein concentrations the curves merge, at both angles, as [poly G] is decreased. This means that in this region the number and size of scattering centers are determined solely by [poly G]; i.e., all poly G is bound, but not all the ribonuclease. Evidence cited below indicates that on the high-[poly G] side of the peaks the situation is reversed; all protein is bound, but not all poly G.

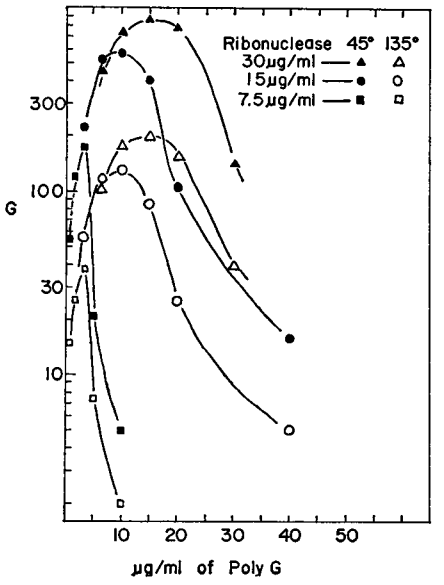


FIGURE 2 Curves of scattered-light intensity vs. poly G concentration, 15 min after ribonuclease addition, in experiments such as those shown in Fig. 1 *a* (these curves are analogous to those involving lysozyme and poly C in Fig. 2 of reference 3).

*Enzymatic Activity of Poly G-Bound Pancreatic Ribonuclease*

Mixtures containing 15 μg of ribonuclease and various weights of poly G per ml were prepared with the buffer. These scattered blue light as depicted by the 15-μg/ml curve of Fig. 2. 15 min after mixing, 0.5 ml of each mixture was added to 3.5 ml of buffer containing 200 μg of poly U per ml. The ensuing hydrolysis was stopped at various times after enzyme substrate contact. The results are shown in Fig. 3. Under the assay conditions employed $OD_{2600A}$ should have been 0.53 if hydrolysis were complete (5). The optical density is plotted against [poly G] in the solution containing 15 μg of ribonuclease per ml. In 3 min the hydrolysis is virtually complete. The 0.5-min curve shows that there is a slight time delay at higher [poly G]'s. However, this is not significant; the binding of pancreatic ribonuclease to poly G does not significantly retard the depolymerization of poly U.

*Complete Ribonuclease Inhibition*

The light-scattering power of 0.1 M acetate buffer (pH 5.4) solutions containing between 5 and 7 μg of poly U and between 14 and 18 μg of lysozyme per ml is exceptionally high; in fact such mixtures appear opalescent when solution light paths are only about 3 cm. They remain like this for long periods of time. Nothing precipitates. They rapidly clarify upon addition of a small quantity of pancreatic ribonuclease, because this enzyme destroys the poly U and lysozyme cannot form large complexes with uridylic acid. This property was used to study the action of

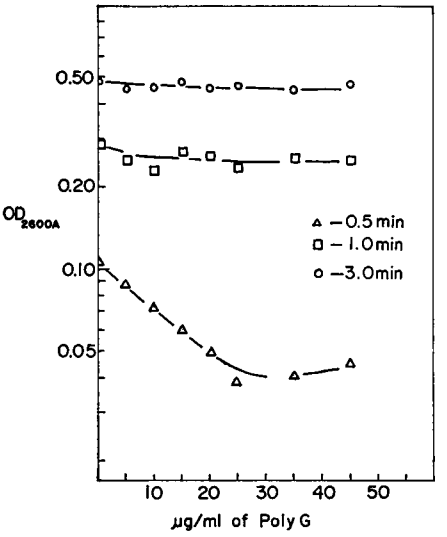


FIGURE 3 Formation of hydrolysis products in solutions of poly U to which pancreatic ribonuclease-poly G mixtures were added. [Ribonuclease]/[poly G] is of the order of 1, as in the 15-μg/ml curve of Fig. 2. Under the conditions employed, complete hydrolysis gives an optical density near 0.53 (see text).

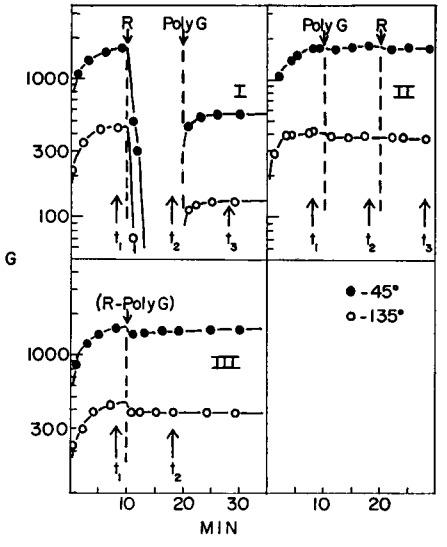


FIGURE 4 Curves of scattered-light intensity vs. time obtained by adding ribonuclease (*R*), poly G or a mixture of these (*R*-poly G) to solutions containing large lysozyme-poly U complexes. Note effects of reversing the order of additions (see text).

pancreatic ribonuclease on lysozyme-bound poly U. One such series of experiments is described here.

Four solutions were made with the buffer. They were

$S_L$ containing 100 μg of lysozyme per ml,

$S_{poly\ U}$ containing 7.5 μg of poly U per ml,

$S_R$ containing 10 μg of ribonuclease per ml and

$S_{poly\ G}$ containing 200 μg of poly G per ml.

At $t = 0$ min 1.8 ml of $S_L$ was added to 8.5 ml of $S_{poly\ U}$ in 25-mm light-scattering

cells. The mixtures become opalescent within 3 min after lysozyme addition. [Lysozyme] was 17.5 $\mu$g/ml and [poly U] was 6.2 $\mu$g/ml. Further additions were made to this mixture as follows:

Experiment I. At $t$ = 10 min 0.7 ml of $S_R$ was added. At $t$ = 20 min 1.4 ml of $S_{poly\ G}$ was added.

Experiment II. At $t$ = 10 min 1.4 ml of $S_{poly\ G}$ was added. At $t$ = 20 min 0.7 ml of $S_R$ was added.

Experiment III. 1 ml of $S_R$ was added to 2 ml of $S_{poly\ G}$. This mixture was allowed to stand for 5 min. At $t$ = 10 min 2.1 ml of this ribonuclease-poly G mixture was added to the solution containing the large lysozyme-poly U complexes.

In all three experiments the final concentrations were [ribonuclease] = 0.56 $\mu$g/ml, [lysozyme] = 14.5 $\mu$g/ml, [poly G] = 22.5 $\mu$g/ml and [poly U] and/or [hydrolysis products] = 5.2 $\mu$g/ml. Results are shown in Fig. 4. It is clear that pancreatic ribonuclease destroys the lysozyme-poly U complexes unless poly G is added before or at the same time. In experiment I the turbidity rises again, after poly G addition, because the lysozyme formerly bound to poly U is now free to form complexes with poly G. Conditions are about right for the largest lysozyme-poly G complexes to form (see Fig. 3 of reference 3).

Final confirmation as to whether or not poly U has been destroyed comes from a determination of the presence or absence of its hydrolysis products. 8 min after each addition step (at times designated by $t_1$, $t_2$, etc. in Fig. 4) aliquots were removed and assayed for hydrolysis products. Under the conditions employed complete hydrolysis should have yielded an $OD_{2600\,A}$ = 0.13. The values obtained are listed in Table II, along with the visible appearance of the mixtures from which aliquots were removed for the assay. We have included a 2-hr measurement to show that intact poly U was still present.

In these experiments [ribonuclease]/[poly G] is about 1/40. According to the re-

TABLE II

HYDROLYSIS PRODUCTS FORMED AND APPEARANCE OF THE MIXTURES AT THE TIMES CITED IN FIGURE 4

| Exp. | Time | Opalescence in room light | Optical density at 2600 A |
|---|---|---|---|
| I | $t_1$ | Strong | 0.025 |
| I | $t_2$ | Absent | 0.123 |
| I | $t_3$ | Very faint | 0.135 |
| II | $t_1$ | Strong | 0.025 |
| II | $t_2$ | Strong | 0.010 |
| II | $t_3$ | Strong | 0.060 |
| III | $t_1$ | Strong | 0.024 |
| III | $t_2$ | Strong | 0.026 |
| III | $t_{2hr}$ | Strong | 0.022 |

TABLE III

PROTECTION OF LYSOZYME-BOUND AND FREE POLY U(SEE TEXT)

| [Poly G] | [Ribonuclease]/[poly G] | With lysozyme | | Without lysozyme | |
|---|---|---|---|---|---|
| | | OD | Appears | OD | Appears |
| *μg/ml* | | | | | |
| 0 | ∞ | 0.148 | Clear | 0.148 | Clear |
| 1.7 | 1/2 | 0.143 | Clear | 0.145 | Clear |
| 3.3 | 1/4 | 0.144 | Clear | 0.150 | Clear |
| 5.0 | 1/6 | 0.135 | Clear | 0.144 | Clear |
| 6.7 | 1/8 | 0.120 | Clear | 0.144 | Clear |
| 10.0 | 1/12 | 0.074 | Weakly turbid | 0.138 | Clear |
| 16.7 | 1/20 | 0.038 | Turbid | 0.121 | Clear |
| 20.0 | 1/24 | 0.040 | Turbid | 0.124 | Clear |
| 26.7 | 1/32 | 0.036 | Turbid | 0.120 | Clear |

sults shown in Fig. 2, this is far more than enough poly G to complex with what ribonuclease is present. Similar experiments were carried out to ascertain the effects of varying this ratio. A fixed quantity of ribonuclease was mixed with various quantities of poly G in the buffer. This was added to buffer solutions containing (*1*) large lysozyme-poly U complexes or (*2*) the same concentration of poly U and no lysozyme. After all components were added [poly U] = 5.8 μg/ml, [ribonuclease] = 0.83 μg/ml, [lysozyme] = 15 (or 0) μg/ml and [poly G] was fixed at a particular value. The mixtures were assayed for hydrolysis products 10 min after ribonuclease-poly U contact. Under the conditions employed complete hydrolysis should have yielded an $OD_{2600A}$ = 0.15. Results are shown in Table III.

It is clear that poly U must be bound to lysozyme and [ribonuclease]/[poly G] must be less than about 1/10 before hydrolysis stops. Further confirmation comes from the visible appearance of the mixtures. However, there was a slight decrease in hydrolysis with increasing [poly G], even when [lysozyme] = 0. For this reason the following experiments were performed.

### *Large excesses of Poly U and Poly G*

3 ml of buffer containing 60 μg of lysozyme and 0.8 μg of ribonuclease per ml was added to 9 ml of buffer containing 89 μg of poly U and various weights of poly G per ml. After protein addition the mixtures contained 67 μg of poly U, 15 μg of lysozyme, 0.2 μg of ribonuclease and various weights of poly G per ml. [Ribonuclease] was low because it was desirable to reduce the rate of poly U hydrolysis. Results are shown in Fig. 5.

When [poly G] = 0, the turbidity rises upon protein addition because [poly U] is reduced, by hydrolysis, from 67 μg/ml toward 6 μg/ml at which the largest lysozyme-poly U complexes are formed. The final fall in scattering occurs because the

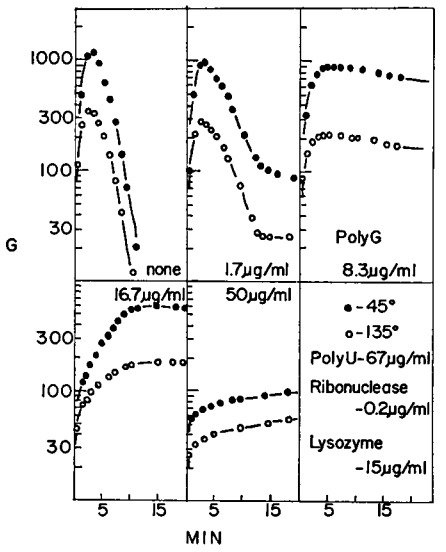


FIGURE 5 Curves of scattered-light intensity vs. time obtained by the simultaneous addition of ribonuclease and lysozyme to solutions containing poly U and various amounts of poly G. Concentrations, just after protein addition, are shown in the figure (see text).

TABLE IV

POLY U REMAINING, 15 MIN AFTER PROTEIN ADDITION (FIG. 5)

| Lysozyme | Poly G | Optical density at 2600 A | Hydrolysis | Remaining poly U |
|---|---|---|---|---|
| *μg/ml* | *μg/ml* | | % | *μg/ml* |
| 15 | 0 | 0.228 | 100 | 0 |
| 15 | 8.3 | 0.194 | 85 | 10 |
| 15 | 50 | 0.158 | 69 | 21 |
| 0 | 8.3 | 0.216 | 93.5 | 4.5 |
| 0 | 50 | 0.173 | 76 | 16 |

ribonuclease is digesting the lysozyme-bound poly U and thereby destroying the complexes. Similar behavior has been observed during the formation and destruction of lysozyme-poly C complexes (see Fig. 4 of reference 3). The other curves show the effects of increasing [poly G]. When [ribonuclease]/[poly G] = 1/100 or less the peak ceases to exist.

Aliquots were removed and assayed for hydrolysis products 15 min after protein addition. Under the conditions employed complete hydrolysis corresponded to an $OD_{2600A}$ = 0.23. Some results are shown in Table IV. Data taken with [lysozyme] = 0 are also included. The remaining [poly U] is greater by about 5 μg/ml when lysozyme is present. This concentration difference persists at high [poly G]'s and reflects the additional protection afforded lysozyme-bound poly U. However, more poly U remains, with or without lysozyme present as [poly G] is increased to

very high values. This type of protection does not last; all the poly U is hydrolyzed within about 1 hr. On the other hand, the lysozyme-bound poly U lasts for much longer periods of time. This was confirmed both by the persistance of a visibly high turbidity and the lack of hydrolysis products.

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