DYNAMICS OF THE INTERACTIONS OF BASIC PROTEINS WITH POLYRIBONUCLEOTIDES

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ABSTRACT Pancreatic ribonuclease was irradiated in the dried state with electrons and then added to acetate buffer solutions that contained different concentrations of polyribonucleotides. Qualitatively similar results were obtained by adding a combination of unirradiated ribonuclease and lysozyme to such solutions. Such solutions scatter light strongly, and the intensity of the scattered light changes with time after mixing. The angular distribution of the scattered light was obtained as a function of time and compared with the rates at which hydrolysis products were formed. The turbidity of the solutions increases rapidly with time at the lower polyribonucleotide concentrations, and seems to result from a complex between inactive ribonuclease, or lysozyme, and oligonucleotides that appear during enzymic hydrolysis of the polynucleotides. The dissymmetry of the scattered light is approximately 5, indicating that the scattering centers are, if spherical, about 1500 A in diameter. The turbidities are remarkably high when one considers the low concentrations of protein and nucleic acid materials that are used.

INTRODUCTION

Pancreatic ribonuclease and other basic proteins, when irradiated or heated in the dried state (1), increase the light-scattering power of polyribonucleotide solutions to which they are added. The rise (or fall) of the turbidity of such solutions can easily be compared, on a radiation dose scale, with the amount of enzymatic activity retained by the added ribonuclease (see bibliography of reference 1). Recently it was shown (2) that the intensity of the scattered light changes with time in a manner that can be related to the rate at which the particular polyribonucleotide present is hydrolyzed by ribonuclease. The entities which give rise to the intense light scattering do not form unless damaged (enzymatically inactive) ribonuclease molecules are present, and this reaction is modified by hydrolysis of the polymers by enzymatically active molecules (which by themselves cannot increase scattering of light).

I have added irradiated ribonuclease to solutions containing different concentrations of polyadenylic acid (poly A), polycytidylic acid (poly C), and polyuridylic acid (poly U). The relative intensity of the light scattered at each of several angles was determined at various times after protein addition. These results were com-

pared with the rates at which hydrolysis products appeared in corresponding solutions. The curves of scattered-light intensity versus time have a well defined maximum (at all scattering angles) when poly C or poly U is involved. It is surprising that such maxima occur at times when enzymatic hydrolysis has virtually ceased. This strongly suggests that the most intense scattering occurs at times when inactive ribonuclease forms a complex with oligonucleotides that fall within some critical range of sizes. In another series of experiments I have added mixtures of intact (unirradiated) ribonuclease and lysozyme to solutions of these homopolymers that were buffered to the same pH. The curves of scattered-light intensity versus time again have a well defined maximum when poly C or poly U is involved. Under the experimental conditions employed, lysozyme by itself does not appreciably alter the turbidity of poly C or poly U solutions to which it is added; enzymatically active ribonuclease must be present.

The angular distribution of the intensity of scattered light must be obtained before anything meaningful can be said about sources of the light scattering. For example, a rise of this intensity at angles near 90° to the transmitted light beam reflects an increase in the number and/or size of such sources. However, a fall at this angle does not necessarily mean the reverse; it may be that the sources have become so large that there is appreciable internal interference. In these experiments dissymmetries can be 5 or greater, indicating that the light is scattered from very large particles.

MATERIALS AND METHODS

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

TABLE I
MATERIALS

Compound	Specifications and supplier
Ribonuclease	Pancreatic, 5× crystallized, lots 50631 and 59616, from General BioChemicals, Chagrin Falls, Ohio.
Lysozyme	3× crystallized, lot 50433, from General Biochemicals
Polyadenylic acid (poly A)	Potassium salt, control numbers 12528 and 17636, from Miles Laboratories, Elkhart, Indiana.
Polycytidylic acid (poly C)	Potassium salt, control numbers 27518 and 212520, from Miles Laboratories
Polyuridylic acid (poly U)	Ammonium salt, control numbers 412436 and 48646, from Miles Laboratories
Oligonucleotide A4, terminal mixture	Chain length 4, control number 4A23, from Miles Laboratories
Oligonucleotide A8, terminal mixture	Chain length 8, control number 8A23, from Miles Laboratories

Irradiation Procedures

20 μg of ribonuclease to be irradiated was desiccator dried from Baxter distilled injection water solutions on each of several ½-inch stainless steel discs. These thin layers were bom-

barded *in vacuo* with 7-kev electrons as previously described (2). In this work the radiation dose was maintained at 1.8×10^{14} electrons/cm^2, since this makes the turbidity of polyribonucleotide solutions, to which the ribonuclease is added, highest. About 15% of the enzymatic activity is retained. All scattering solutions contain the same ratio of damaged to undamaged molecules.

Other experiments and previous data (1) show that almost identical results can be obtained by heating the ribonuclease *in vacuo* for about 50 min at 190°C. Irradiation is much the faster of the two methods; each disc containing the ribonuclease has only to be exposed to a beam current of about 0.64 μamp/cm^2 for about 45 sec.

Light-Scattering Measurements

The irradiated ribonuclease from several stainless-steel discs was dissolved in 0.1 M acetate buffer (pH 5.4) to a final concentration of 20 μg/ml. 3 ml of this solution was pipetted into a 25-ml-diameter cylindrical light-scattering cell, which already contained 9 ml of a polyribonucleotide solution made with the same buffer. Thus each light-scattering cell had 5 μg of irradiated ribonuclease per ml. For experiments not involving the irradiation of ribonuclease, 3 ml of the buffer solution containing 50 μg of lysozyme and 2 μg of ribonuclease per ml was added to the contents of the light-scattering cells; i.e. in these experiments each cell contained 12.5 μg of lysozyme and 0.5 μg of active (unirradiated) ribonuclease per ml of solution.

A Brice-Phoenix photometer was used to obtain all light-scattering data. Its sensitivity was kept constant, and thus all corrected galvanometer deflections shown in the figures are on the same scale; i.e, they are all true intensities multiplied by the same constant. The 4360-A Hg line was used for all measurements. In most cases these were made at angles of 135°, 105°, 75°, and 45° with the transmitted light beam, in that order, at various times after protein addition. Such measurements could, if necessary, be made every 15 sec. This interval was safely larger than the time constant (about 4 sec) of the galvanometer connected to the output of the photomultiplier tube. Furthermore, the measuring slit could be positioned and neutral filters could be put in or removed from the primary light beam within this time interval. The intensity of scattered light changed very rapidly with time at the lower poly C and poly U concentrations. This behavior dictated choice of the 30° angular interval, since only 1 min would elapse between successive measurements at the same angle (15 sec per measurement at each of the four angles). To be sure, one might have added the proteins, recorded the continuous change at one angle, and then successively repeated the procedure at other angles, using aliquots of the same or newly prepared polyribonucleotide solutions. However, this would entail either letting solutions of compounds not noted for their stability stand for rather long periods of time, or using excessive quantities of these compounds, noted for their costliness.

The use of the four angles given above allows one to calculate the dissymmetry and to estimate the integrated scattered-light intensity over all angles.

Galvanometer deflections (G), given in the figures, have been corrected by (a) subtracting from them the deflections obtained with only buffer in the light-scattering cells, (b) dividing each of these by the fraction of light transmitted by the particular neutral filter in the primary beam for that measurement, and (c) multiplying the resulting value by the sine of the angle with the transmitted light beam. This last procedure takes into account the volume of solution viewed through the slit in front of the photomultiplier tube.

Optical Measurement of Hydrolysis Products

Proteins as used in light-scattering experiments were added to aliquots of the same polyribonucleotide solutions. Concentrations of proteins and nucleic acids were identical with those employed in corresponding light-scattering assays, the only difference being that after mixing

of the proteins and nucleic acids the volume of the solution was 4 ml. The reaction was stopped at various times by adding 1 ml of 25% perchloric acid solution, containing 1%, by weight, uranyl acetate. The resulting mixture was spun at 3000 *g* for 10 min, the supernatant fluid was diluted either 5- or 10-fold with distilled water, and the absorbance was determined at 2600 A. In some experiments the enzymic digestion was allowed to proceed until no further hydrolysis products appeared. When the absorbing solutions contained 20 μg of hydrolysis products per ml, optical densities were about 0.59 (poly U digests) and about 0.32 (poly C digests) at this wavelength. Assuming that UMP and CMP have been produced, one obtains the respective molar extinction coefficients 8.8×10^3 and 4.7×10^3. These are in close agreement with values given in the literature (3), a fact which indicates that enzymic hydrolysis of these homopolymers is complete.

RESULTS

Interactions with Poly C

Ribonuclease, given the fixed radiation dose of 1.8×10^{14} electrons/cm^2 (incident energy 7 kev), was added to acetate buffer (pH 5.4) solutions containing selected concentrations of poly C. The concentration of irradiated ribonuclease was always 5 μg/ml. The angular variation of scattering with time, and the rate of appearance of hydrolysis products in corresponding solutions, are shown in Fig. 1.

The higher the poly C concentration, the longer it takes the scattered-light intensity to reach its highest value and the longer it takes for hydrolysis to go to completion. The most intense scattering is observed when hydrolysis is virtually complete. Just before this time, scattering is increasing more rapidly at 45° and 75°

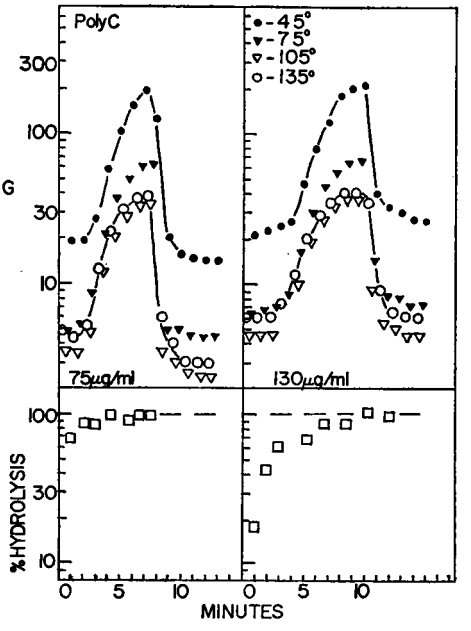

FIGURE 1 Upper curves: the angular distribution of the intensity of light scattered from poly C solutions after irradiated ribonuclease (5 μg/ml) was added. The concentrations of polyribonucleotides are given on these and all following graphs. *G* is the gal. vanometer deflection at each angle and is numerically equal to $2.5 \times 10^5 R$ in which *R* is the Rayleigh ratio at that particular angle. Lower curves: the percentage of complete enzyme hydrolysis at equivalent times (see text).

than at 105° and 135°, indicating that the dimensions of scattering centers are increasing. G_{45}/G_{135} (the dissymmetry) is a commonly used measure of such large-particle internal interference effects. It is about 5 near the scattering peak. G_{75}/G_{135} gives a good visual indication of what is going on; G_{75} and G_{135} are about the same immediately after the irradiated ribonuclease is added, but their curves separate near the scattering peak. Evidently relatively few but quite large scattering centers are still present after the sudden drop in turbidity. I have observed this reaction visually in a darkened room, using a broad beam of green (5460 A) light, which was allowed to pass through a large volume of solution. When turbidity falls, all parts

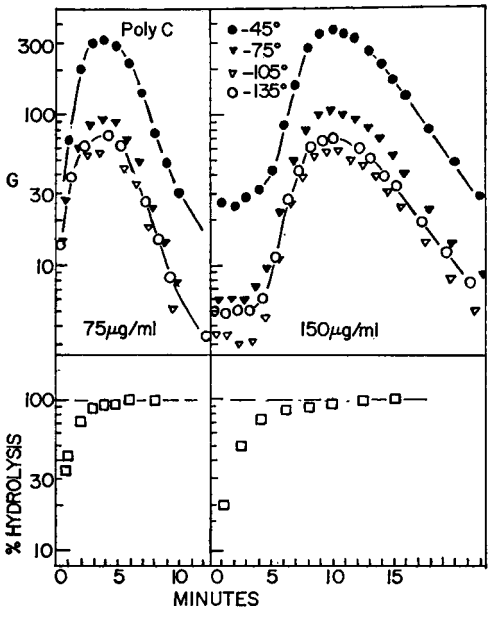

FIGURE 2 The same as Fig. 1 but with unirradiated lysozyme (12.5 μg/ml) and ribonuclease (0.5 μg/ml) added to the poly C solutions.

of the solution clarify at the same rate. Thus, the decrease in turbidity does not seem to be accompanied by a massive precipitation.

The results shown in Fig. 1 strongly suggest that the magnitude of the turbidity rise is especially enhanced when the inactive ribonuclease forms a complex with oligonucleotides that fall within a critical range of sizes. These oligonucleotides are produced during the hydrolysis of poly C by the remaining active ribonuclease. The existence of such oligonucleotides is confirmed by another experiment in which no component is irradiated.

Unirradiated lysozyme does not appreciably increase the turbidity of poly C solutions (pH 5.4) to which it is added, even when present at concentrations up to 30 μg/ml. It does this, however, if before addition it is first mixed with a small amount of active (unirradiated) ribonuclease. The results shown in Fig. 2 were obtained with solutions which, after protein addition, contained 12.5 μg of lysozyme,

0.5 μg of ribonuclease, and the designated weights of poly C per ml of 0.1 M acetate buffer (pH 5.4). These concentrations were chosen because they produce results that most closely match those shown in Fig. 1. Increasing ribonuclease concentration to 1 μg/ml makes the turbidity rise too fast; increasing (decreasing) lysozyme concentration makes the turbidity too high (low). Such changes of protein concentration are not the subject matter of this paper. I only attempt to show here that the light-scattering behavior of a poly C solution containing 12.5 μg of lysozyme and 0.5 μg of ribonuclease per ml is similar to that of a poly C solution containing 5 μg of irradiated ribonuclease per ml.

The results shown in Fig. 2 are much like those in Fig. 1, even in that the dissymmetry approaches 5 near the scattering peak. In other experiments involving the same concentrations of lysozyme, ribonuclease, and poly C, I have first added the lysozyme and waited 12 or more min before adding the ribonuclease. The turbidity does not change until the ribonuclease is added, whereupon the scattered-light intensity starts to change with time just as it does in Fig. 2. If the proteins are added in the reverse order, the turbidity will not rise at all if the delay time is about 12 min or more, presumably because there are no more oligonucleotides left to interact with the lysozyme. In Fig. 2, the final decay of turbidity is exponential with time, and is much less rapid than that in Fig. 1. This indicates that lysozyme-oligonucleotide complexes are more resistant to enzymatic attack by ribonuclease than are inactive ribonuclease-oligonucleotide complexes.

Interactions with Poly U

Experiments like those carried out with poly C were carried out with poly U. Results of adding irradiated ribonuclease to poly U solutions are shown in Fig. 3. Again the most intense scattering occurs at times when hydrolysis is virtually complete. However, the light-scattering curves (for any angle) do not peak as sharply as with poly C, nor is the intensity of the scattered light nearly as high. When mixtures of lysozyme and ribonuclease were added to poly U solutions, results very much like those shown in Fig. 2 were obtained. The rate of hydrolysis seems to be greater for poly U than for poly C. This could account for the fact that the critical solution conditions, which produce the characteristic peak of the poly C light-scattering curve (Fig. 1), are not obtained with poly U.

Interactions with Poly A

The enzyme assay, described above, did not detect acid-soluble hydrolysis products at any time after irradiated ribonuclease was added to solutions containing any concentration of poly A. The turbidity of such solutions does increase with time. However, at poly A concentrations of the same order as the poly C concentrations used in the above experiments, one has to wait hours (not minutes) for the intensity of the scattered light to reach its highest value (see Fig. 8 of reference 2). Therefore the irradiated ribonuclease was added to solutions containing comparatively low concentrations of poly A. Some results are shown in Fig. 4. As with the other

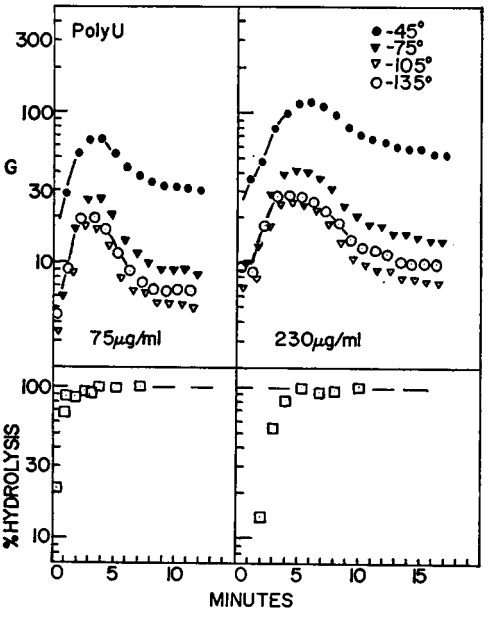

FIGURE 3 Like Fig. 1 but with poly U.

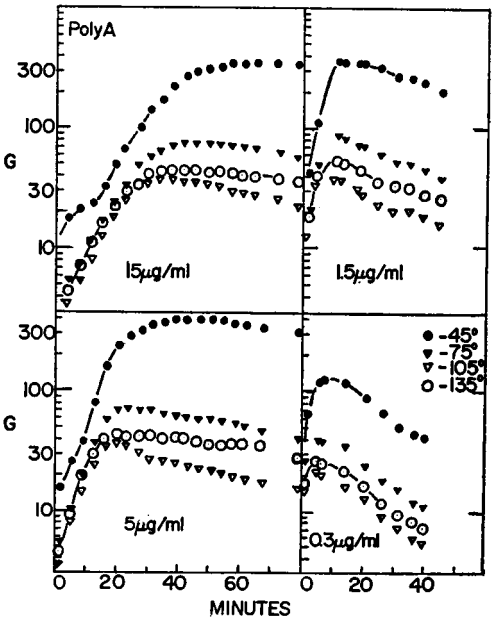

FIGURE 4 Like Fig. 1 but with poly A.

homopolymers, the higher the concentration, the longer it takes for the scattering to reach its highest value. Ratios of intensities at different scattering angles change with time, and are most easily followed at the higher poly A concentrations. Consider the 15 μg/ml curves. G_{45}/G_{135} starts high, decreases, and again rises to a very high value. During these changes both G_{45} and G_{135} are increasing. I interpret such behavior to mean that (*a*) initially, the number of scattering centers is increasing, (*b*) immediately thereafter they are getting smaller, after which (*c*) they again increase in size, to become even larger than they were in the poly C and poly U cases (G_{45}/G_{135} is much larger here). The intermediate decrease in size could be the

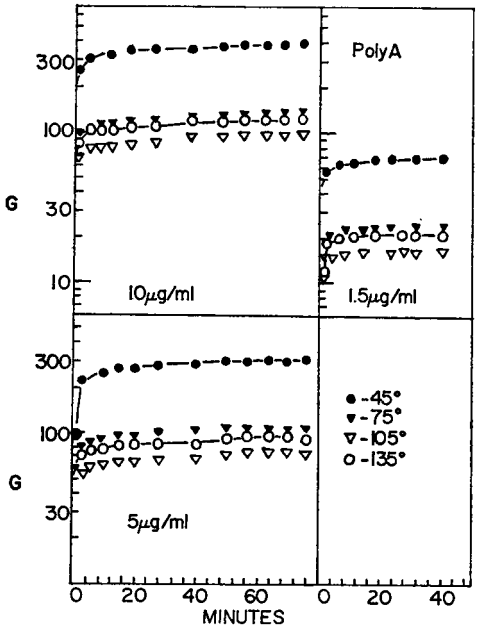

FIGURE 5 Like Fig. 4 but with unirradiated lysozyme (12.5 μg/ml) and ribonuclease (0.5 μg/ml) added to the poly A solutions.

result of partial enzymic hydrolysis, despite the fact that the assay employed could not detect hydrolysis products (this assay detects only oligonucleotides of chain length 5 or so). The curve of G_{105} versus time has, at all concentrations, a maximum at much earlier times than does the curve of G_{45} versus time. Once again G_{75} and G_{135} are initially about the same, but eventually the ratio G_{75}/G_{135} becomes much greater than 1. At 15, 5, and 1.5 μg of poly A per ml the peak value of scattered-light intensity is, at each angle, about the same. Poly A concentration must be less than 1 μg/ml before this fails to be true (see the 0.3 μg/ml curve). Furthermore, the dissymmetry is, for these three concentrations, about the same when G_{45} is highest. At poly A concentrations near 0.1, μg/ml conditions become unstable; in some experiments a turbidity rise is obtained while in others it is not.

Other experiments were carried out in which mixtures of lysozyme and unirradiated ribonuclease were added to solutions containing equivalent concentrations of poly A. Results like those in Fig. 5 were obtained if, after protein addition, the

solutions contained 0.5 μg of ribonuclease, 12.5 μg of lysozyme, and the designated weights of poly A per ml (the same protein concentrations that gave the results with poly C in Fig. 2). Within 5 min the scattered-light intensity rises from a value not much higher than it would have with the buffer itself, to a value not much lower than that it has 1 hr after protein addition. The dissymmetry is only about 3. The same results are obtained by adding the same quantity of lysozyme and no ribonuclease at all. If the lysozyme is added first and the ribonuclease is added at some later time, no change in scattering is observed upon addition of the ribonuclease. If the order of protein addition is reversed, results just like those in Fig. 5 are obtained, provided that the delay before lysozyme addition is not longer than 1 hr. In the latter case the turbidity is slightly higher; this could be the result of a very slow hydrolysis of poly A by the ribonuclease. Such results indicate that lysozyme complexes directly with poly A, and that such complexes are not influenced by the presence of ribonuclease. Contrast this with what happens when the two proteins are added to solutions of poly C. In that case the lysozyme does not complex with poly C, but does with oligonucleotides produced during hydrolysis of the poly C by ribonuclease.

DISCUSSION

The angular distributions of the scattered-light intensity from solutions of poly C that contain either 5 μg of irradiated ribonuclease per ml (Fig. 1) or 12.5 μg of lysozyme and 0.5 μg of ribonuclease per ml (Fig. 2) indicate that the scattering centers are very large. The dissymmetry approaches 5 at the scattering peaks. If such scatterers were spherical, their diameter would have to be $\approx \lambda_{water}/2 \approx 1500$ A (4). This dimension excludes particle weights less than 10^9 for the scatterers, since their densities have to be greater than 1. Evidently, even larger particles are formed, but at a slower rate, in poly A solutions that contain 5 μg of irradiated ribonuclease per ml (Fig. 4). However, the dissymmetry of the light scattered from poly A solutions that contain 12.5 μg of lysozyme and 0.5 μg of ribonuclease per ml is only about 3 (Fig. 5). It would still be 3, and the intensity of the light scattered at all angles would still be the same, if no ribonuclease were present at all. It is clear that in this case lysozyme–poly A complexes are formed without benefit of ribonuclease action. Lysozyme does not form such complexes with poly C or poly U, but forms still larger complexes with their oligonucleotides.

The present assay does not detect hydrolysis products in poly A solutions. However, it has been shown that pancreatic ribonuclease does depolymerize poly A at higher pH's (5). Therefore it may be that the reaction is, at pH 5.4, too slow to reduce hydrolysis products to a detectable size (oligonucleotide chain length of 5 or so). I have added irradiated ribonuclease to 0.1 M acetate buffer solutions (pH 5.4) of adenylate oligonucleotides (chain lengths 4 and 8). No significant rise in turbidity was observed. However, the angular variation of scattered-light intensity with time (Fig. 4) strongly suggests that there is a slow hydrolysis of poly A by

ribonuclease that survives irradiation, and that the scatterers are complexes of inactive ribonuclease and comparatively long adenylate oligonucleotides.

Basic globular proteins have a net positive charge when in solutions buffered to pH 5.4. There are other basic proteins that, when irradiated or heated in the dried state, increase the turbidity of polyribonucleotide solutions (pH 5.4) to which they are added (I have not found an acidic protein which does this, whether first irradiated or not). However, the turbidity is never as high as it is when irradiated ribonuclease is added, presumably because the oligonucleotides which fall within a critical range of sizes are not produced. One exception is lysozyme; this basic protein, without benefit of prior irradiation or heating, produces a slight rise in the turbidity of poly A solutions (pH 5.4) to which it is added, even when present at concentrations of only about 8 μg/ml (12.5 μg/ml for results shown in Fig. 5). Such results, when combined with those described in the present paper, indicate that there are at least three factors that contribute to enhance the light-scattering power of solutions of polyribonucleotides and proteins. These factors need not be simultaneously operative. They are that (1) the net charge on the protein be positive; (2) the configuration of the protein in solution be altered (by irradiating or heating it before addition); (3) the polyribonucleotide by depolymerized to oligonucleotides that fall within a critical range of sizes.

It is clear (*a*) that (1), (2), and (3) are operative when irradiated ribonuclease is added to polyribonucleotide solutions; (*b*) that only (1) and (2) are operative when other irradiated basic proteins are added to such solutions; (*c*) that only (1) is operative when lysozyme (or a mixture of lysozyme and ribonuclease) is added to poly A solutions; and (*d*) that only (1) and (3) are operative when mixtures of unirradiated ribonuclease and lysozyme are added to poly C or poly U solutions.

In 1959 R. B. Setlow (personal communication) suggested to the author that irradiated basic proteins increase the turbidity of RNA solutions to which they are added because they take on, when in solution, a nonglobular configuration which exposes hidden reactive groups. That this is important can be seen by comparing the results shown in Figs. 1 and 2. The former were obtained with only 5 μg/ml of irradiated ribonuclease, whereas the latter were obtained with 12.5 μg of lysozyme and 0.5 μg of unirradiated ribonuclease per ml of solution. In the latter case there would be no measurable rise in turbidity if the lysozyme concentration were only 5 μg/ml. The higher lysozyme concentration is required to get equivalent scattered-light amplitudes because this protein is globular in such solutions.

Absolute Turbidities

All the solutions described above can be clarified by centrifugation at no more than 10,000 *g* for no more than 15 min. This again suggests a particle weight of about 10^9 for the scatterers. They would obviously have to be very large if made up of only nucleic acid and protein. A 1000 μg/ml solution of a globular protein of molecular weight 10^5 has a turbidity roughly equal to about 10^{-3} cm^{-1}. There are two inde-

pendent methods of showing that the turbidities of solutions in the above experiments are at least 5 times as great as this, even when they contain less than one one-hundreth this weight of protein and nucleic acid per ml (see the first two chapters of reference 4).

The ordinate G on the graphs is the observed galvanometer deflection, corrected as described in the Methods section. At the fixed sensitivity of the photometer used in all experiments, the galvanometer deflection G_α at an angle α is approximately $2.5 \times 10^5 R_\alpha$, in which R_α is the Rayleigh ratio. Integration of R_α over a unit sphere gives the turbidity. If light scattering does not change too rapidly with time, this integral may be approximated by a sum. This was done for the 5 μg/ml curve of Fig. 4, at about 40 min after irradiated ribonuclease was added, giving the rough value 7×10^{-3} cm^{-1} for the turbidity. A crude check of this value can be made by taking advantage of the fact that turbidity, if high enough, can be determined by doing a transmission experiment. Repeated measurements with the same poly A solution showed about 97.5% transmission for a 4-cm light path, at about 40 min after the irradiated ribonuclease was added. This corresponds to a turbidity of about 6×10^{-3} cm^{-1}, which is in fair agreement with the above value. The background turbidity of the 0.1 M acetate buffer solution is only about 10^{-4} cm^{-1}.

Nature of the Scattering Centers

The scattering centers have particle weights of the order of 10^9, i.e. they are larger than most viruses. The results obtained here indicate that protein rather than nucleic acid concentration is the limiting factor which determined particle size (see Fig. 4). Such particles are most easily formed when oligonucleotides that fall within a critical range of sizes are present. The results also show that adenylate oligonucleotides that form such complexes are of chain length greater than 8. The scatterers are isotropic; the light scattered at 90° is about 100% polarized. There is no measurable fluorescence. I have run parallel experiments with the solutions in the cells of a differential refractometer and found no change in the index of refraction during the turbidity changes.

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REFERENCES

1. MULLANEY, P. F., and J. W. PREISS. 1966. *Radiation Res.* **27**:582.
2. PREISS, J. W. 1967. *Radiation Res.* **30**:1.
3. STEINER, R. F. 1965. The Chemical Foundations of Molecular Biology. D. Van Nostrand, Princeton, N. J. 264.
4. STACEY, K. A. 1956. Light-Scattering in Physical Chemistry. Academic Press, New York. 63, table.
5. BEERS, R. F., JR. 1960. *J. Biol. Chem.* **235**:2393.