SOME PARALLELISMS IN THE BEHAVIOR OF PANCREATIC RIBONUCLEASE AND CHICKEN LYSOZYME TOWARD HOMOPOLYRIBONUCLEOTIDES

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ABSTRACT Pancreatic ribonuclease and chicken lysozyme possess gross similarities that are responsible for a common ability to form enormous light-scattering centers in cooperation with homopolyribonucleotides. The light-scattering power of the mixtures is highest when [homopolymer]/[protein] assumes some critical value that is unique for each homopolymer-protein pair. In some respects the scatterers resemble very large antigen-antibody networks. A criterion is established to ascertain the relative abilities of the homopolymers to form the centers with the two proteins. Both see polyinosinic acid (poly-I) as most and polyadenylic acid (poly-A) as least efficient in this respect.

INTRODUCTION

Pancreatic ribonuclease (particle weight = 1.37×10^4) and chicken lysozyme (particle weight = 1.46×10^4) are small globular proteins containing 124 and 129 amino acids respectively. Both are basic and both contain four sulfur bridges. The first catalyzes hydrolysis of certain phosphate-ester linkages in polyribonucleotides; the latter is inert in this respect. The gross similarities, however, are responsible for some common behavior in aqueous media. This involves polyribonucleotides; hence, a comparison is of considerable interest.

Both proteins interact with these polymers to form enormous light-scattering centers (particle weights about 10^9) in slightly acidic solutions (1). If the polyribonucleotide is a homopolymer, the light-scattering power of the mixtures is especially high when [homopolymer]/[protein] assumes some critical value (1, 2). Chicken lysozyme interacts in this way with all five of poly-I, polyguanylic acid (poly-G), polyuridylic acid (poly-U), polycytidylic acid (poly-C), and poly-A. Pancreatic ribonuclease does so with poly-I, poly-G and poly-A, but not with poly-U and poly-C because of the very rapid hydrolysis of these. In fact, there is a slow hydrolysis of poly-A that renders poly-A–ribonuclease centers unstable.

This is a property of globular proteins and nucleic acid polymers that have

particle weights greater than about 10^4 (1, 2). For example, the scattering centers readily form in lysozyme-tRNA mixtures (tRNA has a particle weight near 2.5×10^4); but they do not form in lysozyme-oligonucleotide mixtures if the chain length is less than about 10. All the homopolymers of the experiments described below have weight-average particle weights $> 10^5$ (according to both the supplier and light-scattering data we have obtained).

All enzymes are globular proteins with particle weights greater than about 10^4. Here we are comparing a common property of two globular proteins, the ability to form large low-density structures in cooperation with polyribonucleotides. Only one has the refinement associated with subsequent (or simultaneous) enzyme chemistry, but the common behavior may be related to the fact that all enzymes are globular proteins. To date no sound physical explanation of this fact exists.

MATERIALS AND METHODS

Specifications, suppliers and notations used for all compounds are listed in Table I. Henceforth these symbols will be employed in the text.

Buffer strength, pH, and temperature (26°C in the photometer) were maintained in all experiments. Each homopolymer assumes the configuration it has under these conditions.

Light-Scattering Measurements

All components were dispersed in 0.1 M acetate buffer (pH 5.4). 1.8 ml of buffer containing 100 μg of ribonuclease (R) or lysozyme (L)/ml was added to 10.2 ml of the same buffer

TABLE I

MATERIALS

Compound	Specifications and suppliers
Polyinosinic acid (poly-I)	Potassium salt, control numbers 11-30-307 and 11-37-307 from Miles Laboratories, Inc., Elkhart, Indiana.
Polyguanylic acid (poly-G)	Sodium salt, control numbers 5272 and 11-06-314 from Miles Laboratories, Inc.
Polyuridylic acid (poly-U)	Ammonium salt, control numbers 41855, 48646 and 45858 from Miles Laboratories, Inc.
Polycytidylic acid (poly-C)	Potassium salt, control numbers 212726 and 27829 from Miles Laboratories, Inc.
Polyadenylic acid (poly-A)	Potassium salt, control number 110638 from Miles Laboratories, Inc.
Pancreatic ribonuclease (R)	5× crystallized, lot 59616 from General Biochemicals, Chagrin Falls, Ohio.
Chicken lysozyme (L)	3× crystallized, lot 50433 from General Biochemicals.

containing various quantities of homopolymer. After this step the mixtures contained 15 μg of L (or R), various weights of one homopolymer (H_i), and 100 (or 0) μg of still another homopolymer (H_j)/ml. The light scattered at 45 and 130° with the transmitted light beam was measured as a function of time after protein addition. This varies little after 5 min within mixing; hence only the 15 min data are shown here (1). There was a slow, steady drop in scattering power of R–poly-I mixtures; this was not significant within the times required to carry out these experiments. The ordinate (G) on the figures is, after correcting for dissymmetry, numerically equal to about $2.1 \times 10^5 R$, in which R is the Rayleigh ratio (1, 3). This facilitates rapid comparison with earlier work (1, 2). The 4358 A Hg line, obtained with a Brice-Phoenix Light-Scattering Photometer (Phoenix Precision Instrument Company, Philadelphia, Pa.), was always employed.

Measurements of Hydrolysis Products

These data were obtained as previously described (1). The assay depends on the measurement of the absorbance of acid-soluble hydrolysis products at 2600 A.

RESULTS AND DISCUSSION

The upper curves of Figs. 1 *a*–1 *e* show the scattering at 45 and 135°, 15 min after mixing lysozyme (L) and the designated homopolymers (H_i). [L] = 15 μg/ml and [H_i] assumes the abscissa values (i = I, G, U, C, or A). All the functions $G([H_i])$ have a maximum at which the dissymmetry (G_{45}/G_{135}) is relatively high. G varies strongly with [H_i] in this region, but is much lower and rather insensitive to large changes in [H_i] near [H_i] = 100 μg/ml.

If the corresponding experiments are carried out with pancreatic ribonuclease (R), one obtains the results shown in the upper part of Fig. 2. Such curves cannot be obtained for i = U, C, or A. R–poly-A scattering centers are unstable (see Figs. 1 *a* and 1 *b* of reference 1) because of slow hydrolysis; R–poly-U and R–poly-C

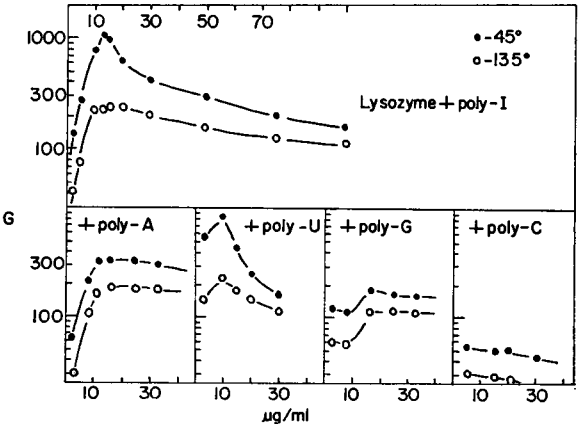

FIGURE 1 *a* Upper curves, scattered light intensity vs. [poly-I], 15 min after adding lysozyme. Lower curves, obtained as were the above, but the mixtures contain 100 μg of the designated competing homopolymer/ml.

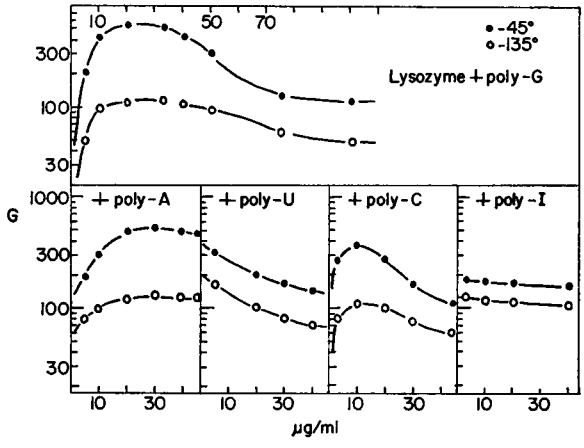

FIGURE 1 *b* Like Fig. 1 *a* but with [poly-G] the variable.

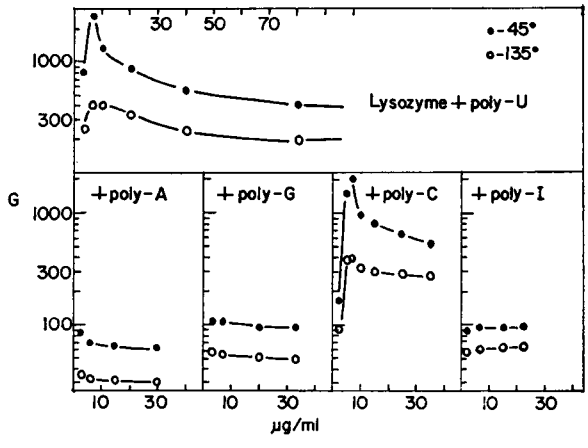

FIGURE 1 *c* Like Fig. 1 *a* but with [poly-U] the variable.

scattering centers either do not form or do not last long enough to be detected because of rapid hydrolysis. There is no large change in G_{45}/G_{135} in the neighborhood of the R–poly-G maximum. We have maintained the same abscissa scale for the R–poly-I curve; this emphasizes the drastically inverted relationship between scattering power and [poly-I]. One might falsely conclude that, at high values of [poly-I], the scattering drops because of weakness or absence of R–poly-I binding. Experiments like the following show that this is indeed not the case.

R and poly-I (or poly-G) were incubated in the buffer for ≥ 5 min; [R] = 15 μg/ml and [poly-I] (or [poly-G]) = 22.5 μg/ml. Aliquots of this mixture were added to the same buffer containing poly-C as substrate. After this step the mixtures contained 1.9 μg of R, 2.8 μg of poly-I (or poly-G), and 175 μg of poly-C/ml. Hydrolysis was stopped at various times and the absorbance of hydrolysis products at 2600 A

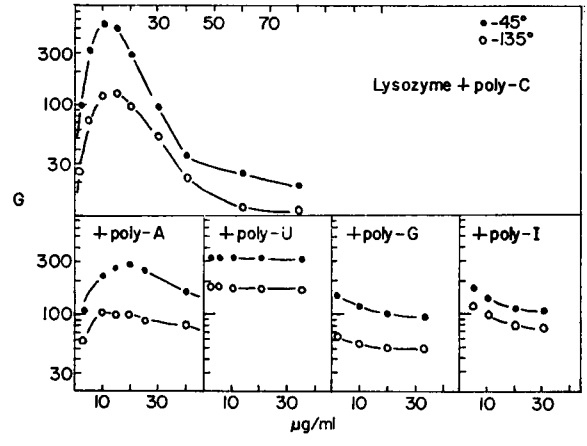

FIGURE 1 *d* Like Fig. 1 *a* but with [poly-C] the variable.

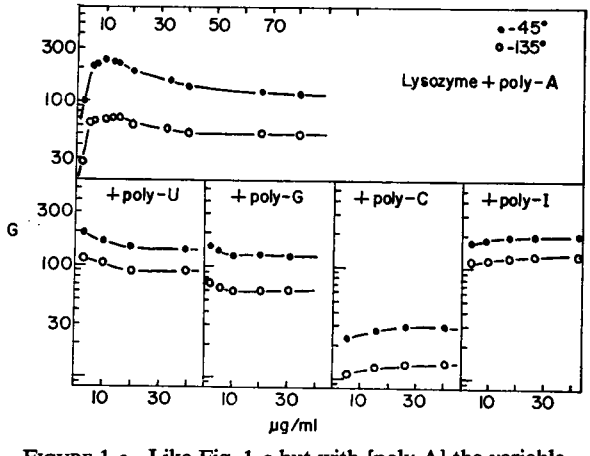

FIGURE 1 *e* Like Fig. 1 *a* but with [poly-A] the variable.

was determined as previously described (1). The results of such a series of experiments are shown in Fig. 3. 100% hydrolysis corresponds to OD 2600 A = 0.20. Clearly, poly-I is a much better inhibitor than is poly-G.

Obviously 2.8 μg of poly-I could not influence 1.9 μg of R in the presence of 175 μg of poly-C/ml without strong R–poly-I binding. Poly-I–poly-C binding is ruled out as an inhibitory factor because there is not enough poly-I to interact with all the poly-C. Similar results were obtained with poly-U as substrate. Note that [poly-G]/[R] is slightly more than two times its value at the R–poly-G peak of Fig. 2; but [poly-I]/[R] is about five times its value at the R–poly-I peak of the same figure. If the same experiments are carried out at the peaking ratios, there is no significant inhibition by either poly-I or poly-G. Hence, R–poly-I binding where scattering power is high is much weaker, with regard to impeding the digestion of an external substrate, than it is when scattering centers do not form. Earlier work

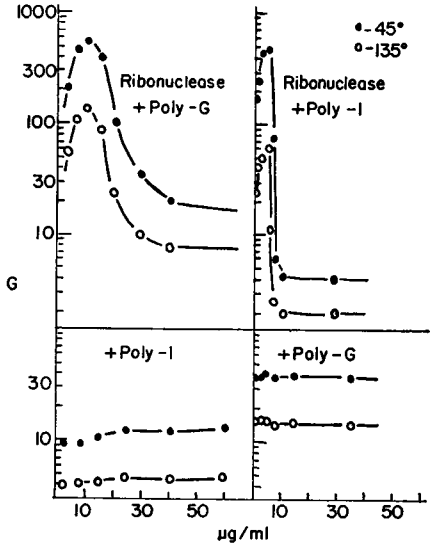

FIGURE 2

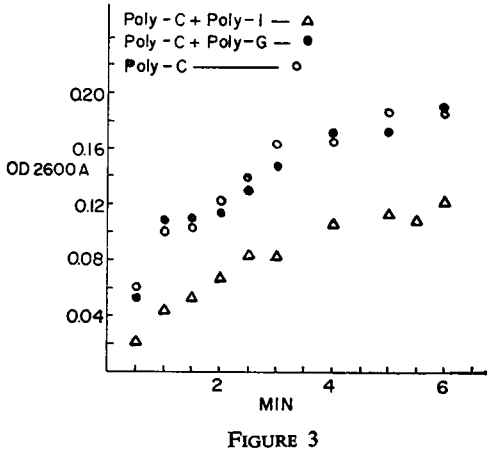

FIGURE 3

FIGURE 2 Upper curves, like the upper curves of Figs. 1 *a* and 1 *b* but with ribonuclease substituted for the lysozyme. Lower curves, obtained as were the upper curves, but the mixtures contain 100 µg of the designated competing homopolymer/ml.

FIGURE 3 The poly-C hydrolysis is impeded when [poly-I]/[ribonuclease] is about 1.5 but not when [poly-G]/[ribonuclease] assumes this same value. There is no impedance when these ratios assume the values they have at the peaks of the upper curves of Fig. 2. In this sense, the binding that causes the scattering centers to form is very weak.

(1) showed that the substrates poly-U and poly-C were easily accessible to R when they were bound in large L–poly-U or L–poly-C scattering centers. Apparently the scattering centers are very large open networks (see Appendix). The concentration sensitivity suggests at least a superficial resemblance to antigen-antibody complexes.

Ordering the Reactions

If the two proteins are to be properly compared, one must find out whether or not each has a homopolymer preference order in forming the large scattering centers; these orders, if they exist, can then be compared.

In the first attempt to accomplish this, L was added to mixtures containing the same concentration (x) of two homopolymers H_i and H_j. x was varied from mixture to mixture. The scattering power of these L-H_i-H_j solutions was highest at one particular x, i.e., the individual L-H_i and L-H_j maxima could not be resolved; however, the light-scattering power of such L–poly-A–poly-U mixtures was unusually low ($G_{45} < 70$). Similar but less pronounced behavior was observed with the L–poly-I–poly-C combination ($G_{45} < 450$ for a peak at $x = 6$ µg/ml). The major difference between these and the other combinations is that there is a possibility of complementary base pairing. Poly-G is already doubly or triply stranded under

these conditions. This is probably why scattering from L–poly-G–poly-C mixtures was just as high as from L–poly-G or L–poly-C mixtures.

In other experiments large L-H_i scattering centers were first formed and the [H_i] or [H_j] of another homopolymer was increased. The expected dramatic drop in scattering did not occur. Evidently the centers, once formed, resist the influence of added homopolymer. Their size is primarily determined by the density of homopolymer strands which the protein sees upon commencement of the reaction. For this reason, the following series of experiments was considered to provide a more elegant test.

Notice that the scattering power of the mixtures is much lower at [H_i] = 100 μg/ml than it is at the peaks (upper curves of Figs. 1 *a*–1 *e* and Fig. 2). In this respect the H_i's might be said to be "self quenching"; however, "cross-quenching" might exist. Hence, a series of experiments was designed in which L or R was added to solutions containing various amounts of one homopolymer H_i and a fixed high quantity of still another H_j. Results are shown in the bottom graphs of Figs. 1 *a*–1 *e* and Fig. 2. After protein addition [L] (or [R]) = 15 μg/ml, [H_i] assumes the abscissa values (as in the upper curves) and [H_j] = 100 μg/ml. Clearly L-H_i-H_j and R-H_i-H_j mixtures could allow reactions that do not occur in L-H_i or R-H_i mixtures; however, the first step is to determine whether or not the variation of [H_i] can still be detected, 15 min after protein addition, as in the upper curves. The latter suggest a criterion for detection. It is that

$$dG/d\,[H_i] = 0 \text{ at some } [H_i] \text{ with } [H_j] = 100\ \mu\text{g/ml}. \qquad (1)$$

This is stronger than a criterion that only looks at the changed shape of a curve, because the vanishing derivative denotes a unique condition brought about only by changing [H_i] against the competing [H_j] = 100 μg/ml.

First consider the reactions with L (lower curves of Figs. 1 *a*–1 *e*). In some cases the variation of [H_i] shows up as well as it does in the absence of H_j (see Fig. 1 *c* with H_i = poly-U and H_j = poly-C). In others the criterion is met but the shape of the curve is not at all like that obtained in the absence of H_j (see Fig. 1 *a* with H_i = poly-I and H_j = poly-A). In still others H_j is completely dominant (see Fig. 1 *d* with H_i = poly-C and H_j = poly-U). In order to unscramble these results, the following simple device was employed.

The symbol $>(<)$ is written when the criterion for detecting [H_i] variation is (is not) satisfied. A 25-member array, in which rows are H_i's and columns are H_j's, is constructed (see Fig. 4). The ordering I-G-U-C-A has the unique feature of making all entries $<$ to the left of the diagonal (along which $i = j$). This also makes all entries $>$ to the right of the diagonal, save for the three circled anomalies; hence the array misses "skew symmetry" by only 3 of 20 off-diagonal entries, and is thus significant despite the anomalies. At least one, however, and possibly all three of the latter can be accounted for.

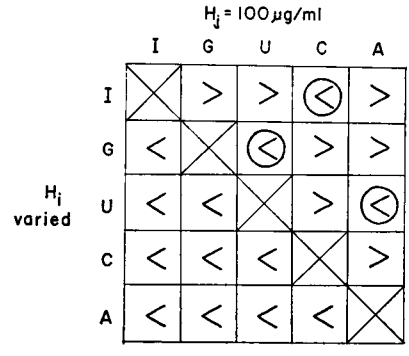

FIGURE 4 The inequality array. [H_i] is the varied concentration and [H_j] is fixed at 100 μg/ml. If variation of the former shows up (according to the criterion established in the text), the symbol > is entered at row H_i, column H_j. If it does not < is entered. $i = j$ along the diagonal. Note the near "anti-symmetry." The circled anomalies are accounted for in the text.

The G = i, U = j and the U = i, A = j anomalies may be considered together. As described above, the light-scattering power of L–poly-U–poly-A mixtures was always very low when [poly-U] = [poly-A]. This is not likely to be due to anything other than A-U base pairing, and thus accounts for the U = i, A = j anomaly. If this is taken into account, we could have inverted the order of G and U in the above sequence and still obtained a U = i, G = j anomaly; hence, our criterion cannot really distinguish U from G in the sequence, and this is why we had the original G = i, U = j anomaly.

As reported above, the light-scattering power of L–poly-I–poly-C mixtures, containing the same concentration of both homopolymers, is very much lower than that obtained with L–poly-I or L–poly-C mixtures, however, there is a moderately strong scattering peak at [poly-I] = [poly-C] = about 6 μg/ml. Thus, I-C base pairing, if it did occur, did not stop the reaction with L. Hence, a base-pairing explanation of the I = i, j = C anomaly is somewhat less tenable than a similar explanation for the U = i, A = j anomaly.

In any case, even without any accounting for the anomalies, the criterion has provided the order

$$\mathrm{I} > \mathrm{G} \approx \mathrm{U} > \mathrm{C} > \mathrm{A} \text{ (for L)}. \qquad (2)$$

Consider the corresponding experiments with R (lower curves of Fig. 2). It is clear that the criterion is not satisfied for either H_i = poly-G, H_j = poly-I, or H_i = poly-I, H_j = poly-G. The added R does not see the [H_i] variation of either homopolymer in the presence of [H_j] = 100 μg/ml of the other. We may take advantage of the fact that hydrolysis of poly-A is very slow (1, 4) under these conditions and vary [H_i] = [poly-I] (or [poly-G]) in the presence of [H_j] = [poly-A] = 100 μg/ml. The resulting curves are hardly different from those in the upper part of the figure.

In the reverse experiments with $[H_i]$ = [poly-A] and $[H_j]$ = [poly-I](or [poly-G]), no large scattering centers form at any [poly-A]. Hence, if we again adopt the symbolic scheme employed in the experiments with L, we obtain the order

$$I \approx G > A \text{ (for R)}. \tag{3}$$

Before considering the significance of the orders 2 and 3 we would here point out a striking similarity to another order obtained by Fujita et al. (5) on the basis of very different considerations. These investigators sought a physical explanation for the degeneracy in the genetic code. This involves the codon of mRNA, the anti-codon of tRNA, and the tautomeric conversion of the base, in the first position of the anti-codon, from enol to keto form. Conversion feasibility is highest for the base I and decreases according to I > G > U > C > A (see the table of reference 5). Hence there is a high probability that the base chemistry of these investigators has much to do with the selective reactions of the homopolymers.

Lysozyme vs. Ribonuclease

One should first recall how the giant scattering centers formed if these proteins are to be compared via such reactions.

(*a*) The size and/or number per unit volume of scattering centers are critically dependent on [homopolymer]/[protein] at the time proteins and homopolymers are mixed. In this sense the centers resemble very large antigen-antibody networks. An analysis based on interference optics confirms this (see Appendix).

(*b*) The size of L-H_i or R-H_i complexes is not a measure of L-H_i or R-H_i binding strength; in fact, the binding at higher $[H_i]$'s, at which the centers do not form, is more effective in impeding the hydrolytic action of R (see the R–poly-I experiments and reference 1). The dissymmetry and amplitude of the scattering both rise very rapidly for spherical or random coil scatterers, once their largest dimension exceeds 1000 A (3). The L–poly-U peak is very high under these conditions; however the poly-U in these complexes is easily accessible to pancreatic ribonuclease (1). (Present experiments show that the L–poly-C peak is, at 37°C, nearly as high as the L–poly-U peak at 26°C.)

(*c*) When the proteins are added to solutions containing two homopolymers H_i and H_j, formation of scattering centers may critically depend on $[H_i]$ despite a high $[H_j]$. If this occurs, it never does when the roles of H_i and H_j are reversed; hence, H_i is superior to H_j with regard to locking the proteins in antigen-antibody–like networks. The important point is that *both* R and L see poly-I as most efficient, and poly-A as least efficient in this respect (see the orders 2 and 3 above). The reactions with R are summarized in Table II according to the order obtained with L. We strongly suspect that R–poly-U and R–poly-C scattering centers may form and break up too fast to be detected by our present equipment. Purine-pyrimidine differences seem to be vary important.

TABLE II

INTERACTIONS WITH PANCREATIC RIBONUCLEASE

Polyribo-nucleotide	Big scattering centers	Hydrolysis	Base
Poly-I	Stable	Absent	Purine
Poly-G	Stable	Absent	Purine
Poly-U	Not detected	Very fast	Pyrimidine
Poly-C	Not detected	Very fast	Pyrimidine
Poly-A	Unstable	Very slow	Purine

(*d*) The R–poly-G and R–poly-I maxima are more critical than are the corresponding maxima obtained with L. In addition, the curves obtained with R do not display the large dissymmetry changes one sees in the corresponding L curves. A dissymmetry change reflects a change in shape and/or size of the scatterers. Apparently neither of these vary in the neighborhood of the maxima obtained with R. Evidently R–poly-G and R–poly-I complexing is geometrically more refined than is the corresponding complexing with L.

On Specificity

If each of five unlabeled bottles contained a given concentration of one of the five homopolymers employed above, one could easily identify all five homopolymers by removing fixed aliquots, mixing with chicken lysozyme, and carrying out the light-scattering experiments described in this and earlier reports (1, 2). On the other hand, if the test protein had been pancreatic ribonuclease, poly-U and poly-C would be indistinguishable without further chemical tests, because the scattering power of hydrolysis products is negligible. Hence, in this one sense, chicken lysozyme is more "specific" than is pancreatic ribonuclease because it *does not* possess the active site of the latter. The common properties of the two stem from their being similar globular proteins. We feel that experiments, designed on that assumption that "specificity" is only to be associated with active sites, cannot explain why all enzymes are globular proteins with particle weights greater than about 10^4.

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APPENDIX

Optical Properties of the Scattering Centers

The light-scattering power of R–poly-G and L–poly-C mixtures is highest when they contain 15 μg of protein and about 10 μg of homopolymer/ml of buffer. The scattering of these

mixtures is here compared with that of a suspension containing 25 μg of tobacco mosaic virus (TMV)/ml of buffer. The particle weight and shape of this virus are well-known.

A very pure TMV preparation was obtained from S. Srinivasan of the University of Pittsburgh. At 25 μg of this virus/ml of buffer, $G_{135} = 48.5$, $G_{105} = 41$, $G_{75} = 48$, and $G_{45} = 105$; $G_{45}/G_{135} = 2.18$, corresponding to a rod of length about 80% the wavelength of the exciting light, in the medium. The reciprocal particle-scattering factors were obtained for this rod (3). These correct for dissymmetry and restore the Rayleigh pattern (symmetrical about 90°). The corrected values are $G_{135,c} = 149$, $G_{105,c} = 106$, $G_{75,c} = 101$, and $G_{45,c} = 148$. These fit the $1 + \cos^2\alpha$ pattern very well.

If the scatterers are small enough, one can choose the set of reciprocal particle-scattering factors that best convert the angular intensity distribution to the Rayleigh pattern, but if the scatterers are large one in general has to account for refraction at the particle-medium interface (3). Distortion due to this effect will be small if the index of refraction of the scatterer is not markedly different from that of the medium (i.e., if the scatterers are of low density).

This is at best a qualitative analysis. Two methods will be employed. An extrapolation will be made to low angles where internal interference is minimal and the reciprocal particle-scattering factors for two models will be applied.

The intensity of the light scattered at a particular angle α is

$$G_\alpha = k_\alpha(\mathrm{d}n/\mathrm{d}c)^2cM, \tag{A 1}$$

in which k_α is a constant for a given wavelength, α, and medium, $\mathrm{d}n/\mathrm{d}c$ is the specific refractive index increment, c is the weight concentration of scatterers, and M is the weight-average molecular weight (3). A rough extrapolation to $\alpha = 0°$, for the scattering from TMV, R-poly-G, and L-poly-C mixtures (at the same c) showed that

$$G_{0,s} \approx 15G_{0,v} \tag{A 2}$$

in which s means scattering center and v means virus. Hence, from equations A 1

$$(\mathrm{d}n/\mathrm{d}c_s)^2c_sM_s \approx 15(\mathrm{d}n/\mathrm{d}c_v)^2c_vM_v\,. \tag{A 3}$$

c_s and c_v are both assumed to be 25 μg/ml. $\mathrm{d}n/\mathrm{d}c_v$ was easily determined to be 0.175 ml/g in this buffer. A Brice-Phoenix differential refractometer was employed, but $\mathrm{d}n/\mathrm{d}c_s$ could not be determined by raising c_s (while maintaining [homopolymer]/[protein] in the peak region) because the mixtures became hopelessly turbid before c_s reached 1 mg/ml. At other higher values of [homopolymer]/[protein], however, it was possible to get c_s up to 2.5 mg/ml (for accurate determinations one ought to get to higher values). In this way the rough value $\mathrm{d}n/\mathrm{d}c_s = 0.18 \pm 0.03$ ml/g was obtained. Within experimental error this is the same as $\mathrm{d}n/\mathrm{d}c_v$. Hence, from equation A 3

$$M_s \approx 15M_v = 0.6 \times 10^9. \tag{A 4}$$

Since this is a weight-average value, any polydispersity requires the presence of still larger scattering centers.

In an alternative approach one can (*a*) assume that distortion due to refraction at the scatterer-medium interface is small and (*b*) apply models that are commensurate with the high dissymmetry. $G_{135} = 130$, $G_{105} = 103$, $G_{75} = 174$, and $G_{45} = 580$ at the R-poly-G peak. $G_{45}/G_{135} = 4.4$. The pattern is very similar for the L-poly-C peak.

This dissymmetry corresponds to spheres of diameter 1500 A and random coils of rms coil length just greater than the wavelength of the light in the medium (about 3300 A). When the appropriate spherical reciprocal scattering factors are applied one obtains $G_{135,c} = 644$, $G_{105,c} = 322$, $G_{75,c} = 332$, and $G_{45,c} = 720$. The corrected values at 105 and 75° are much too low to fit a Rayleigh pattern. The random coil is much better. One obtains $G_{135,c} = 1500$, $G_{105,c} = 900$, $G_{75,c} = 940$, and $G_{45,c} = 1480$. These fit a $1 + \cos^2\alpha$ pattern much better than do the spherical model values. Comparing with the corrected TMV scattering at 45 and 135°, and using equation A 1 we find that

$$M_s \approx 10M_v = 0.4 \times 10^9. \qquad (A\,5)$$

Polydispersity would require the presence of still larger particles; this is weight-average optics.

The weight-average molecular weight of the homopolymers is, according to the supplier, greater than about 10^5. Hence, the average length is of the order of 1000 A. Apparently these join with the proteins in the large scattering centers to form very large open networks of low index of refraction (if the index of refraction had not been low we would not have been able to apply the reciprocal particle-scattering factors for any model). The effective particle weights must be greater than the values in equations A 4 and A 5, because the centers necessarily have to contain medium.

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