TEMPERATURE VARIATION OF POLYRIBONUCLEOTIDE CONFORMATION BY AN INTERACTION WITH BASIC GLOBULAR PROTEINS

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ABSTRACT Chicken lysozyme interacts with polyribonucleotides to form large light-scattering centers. The size of these is critically dependent on conformation of the polynucleotides. Hence, the interaction provides a very sensitive method of determining temperature intervals within which the polynucleotides change from multistranded, stacked-base structures to single-stranded, random coils. This change can be observed with the unaided eye, especially when concentrations of polynucleotides are of the order of a few micrograms per milliliter.

INTRODUCTION

In this paper we introduce a very sensitive method of observing temperature-dependent changes in the configuration of polyribonucleotides. In fact, some effects can be observed with the unaided eye, at polynucleotide concentrations of less than 5 μg/ml.

The method exploits a phenomenon we have reported several times in this journal (1, 2). Some basic proteins (such as chicken lysozyme and pancreatic ribonuclease) interact with polyribonucleotides to form enormous light-scattering centers. If the polynucleotide is a homopolymer, the light-scattering power of the mixtures is especially high when [homopolynucleotide]/[protein] assumes some critical value (2). This inverted relationship between light-scattering power and [homopolymer] (at fixed [protein], pH, and buffer molarity) shows that spacing of reactants is critical; hence, we reasoned that such spacing should in turn be critically dependent on the configurations of interactants. In the temperature range 10–60°C chicken lysozyme and pancreatic ribonuclease retain their globular form. However, the homopolyribonucleotides (poly G excepted), the Watson-Crick pairs (poly U-poly A and poly I-poly C), and yeast tRNA undergo configuration changes, including what has

been called "melting." When this occurs (usually within a much narrower temperature region) there is a drastic increase in the size of lysozyme-polynucleotide light-scattering centers. This is accompanied by an increase in the scattered light intensity of over 1,000% (which can be seen with the unaided eye in a test tube). These should be compared with the 15–30% changes in optical density that currently provide the accepted spectroscopic criteria for such changes.

MATERIALS AND METHODS

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

Light-Scattering Measurements

Stock solutions of the appropriate protein (chicken lysozyme, L, or pancreatic ribonuclease, R) or polyribonucleotide were prepared in 0.1 M acetate buffer (pH 5.5) at a concentration of 100 μg/ml. Samples of the polyribonucleotide stock solution were mixed with the appropriate amount of buffer, totaling 10.2 ml of solution in a 26 mm cylindrical light-scattering cell, to obtain the desired final concentration of the polyribonucleotide. The light-scattering cell was immersed in a constant temperature bath for 5 min in order to raise the temperature of the solution to the desired fixed temperature. The cell was then placed on a cored cell holder inside a Brice-Phoenix Universal Light Scattering Photometer (series 1999-96, Phoenix Precision Instrument Div., Virtis Co., Inc., Gardiner, N. Y.). The temperature of the buffered solution inside the light-scattering cell was maintained by the circulation of water from a constant temperature bath through the cored cell holder. Temperatures of the solution could be maintained to within 0.3°C over a period of at least 15 h. The temperature of a sample of the protein (L or R) stock solution was raised to the desired level, and 1.8 ml

TABLE I
MATERIALS

Compound	Specification and suppliers
Polyinosinic acid (poly I, H_I)	Potassium salt, control nos. 11-30-307 and 11-37-307 from Miles Research Div., Miles Laboratories, Inc., Elkhart, Ind.
Polyguanylic acid (poly G, H_G)	Sodium salt, control numbers 11-06-314 and 11-17-314 from Miles Laboratories, Inc.
Polyuridylic acid (poly U, H_u)	Ammonium salt, control number 11-78-308 from Miles Laboratories, Inc.
Polycytidylic acid (poly C, H_c)	Potassium salt, control number 11-34-304 from Miles Laboratories, Inc.
Polyadenylic acid (poly A, H_A)	Potassium salt, control number 110638 from Miles Laboratories, Inc.
Transfer RNA (tRNA)	Yeast, control number 641605 from General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio
Pancreatic ribonuclease (R)	5 × crystallized, lots 59616 and 83212, from General Biochemicals
Chicken lysozyme (L)	3 × crystallized, lot 50433, from General Biochemicals

was added to the 10.2 ml of the same buffer containing various quantities of the polyribonucleotide 8 min after the light-scattering cell had been placed on the cored cell holder. After this step the mixtures contained 15 μg/ml of L (or R) and various concentrations of one of the polyribonucleotides. The light scattered at 45 and 135° with the transmitted beam was measured as a function of time after protein addition. In most cases the scattering intensity varied very little after about 5 min after mixing. Hence, the 15 min data will be used almost exclusively in this work.

Sudden temperature variations of the solutions of the proteins and polyribonucleotides were investigated at 15 min after the protein addition. The interaction was allowed to proceed for 15 min at 15°C (37°C). At 15 min after protein addition the light-scattering cell was immersed in a constant temperature bath for 2 min to bring the temperature up (down) to 37°C (15°C). The cell was then placed back onto the cored cell holder in which the temperature of the circulating water had been adjusted to maintain the latter temperature. The light scattered was recorded as a function of time at angles of 45 and 135° with the incident beam.

Watson-Crick base pairing (A-U and I-C) was investigated by adding samples of the stock solutions of the homopolymers poly A (poly I) and poly U (poly C) to the buffer as previously described. The final concentrations of the components were: 6.0 μg/ml of poly U (or poly C), 6.5 μg/ml of poly A (or poly I), and 15 μg/ml of L. These mixtures were allowed to stand for at least 5 min at room temperature before proceeding.

The ordinate (G) on the figures is numerically equal to about $2.1 \times 10^5 R$ in which R is the Rayleigh ratio. The 4,358 Å Hg line was always used.

RESULTS AND DISCUSSION

In earlier work (1) we compared the interactions of chicken lysozyme (L) and pancreatic ribonuclease (R) with poly I, poly G, poly U, poly C, and poly A, under conditions identical with those employed in the present experiments (0.1 M acetate buffer, pH 5.5). The temperature was maintained at 26°C. The order that was thereby obtained had nothing to do with the magnitude of the scattered light intensity, which was highest for L-poly U mixtures. We suspected that this was a consequence of poly U existing as single strands at 26°C. The results obtained here show that such intense light scattering is observed with any L-polyribonucleotide mixture at temperatures at or above the melting point of that particular polyribonucleotide.

For example, we may compare temperature effects on L-poly A and L-poly G mixtures. Fig. 1 shows the light scattered at angles of 45 and 135° to the transmitted light beam 15 min after mixing L with these homopolymers. Results are shown at three temperatures. After mixing, the solutions contained 15 μg of L and the designated weights of each homopolymer per milliliter. The L-poly A curves obtained at 10.4°C are only slightly lower than those obtained earlier (1) at 26°C. At 37.4°C a sharp peak has appeared at [poly A] = 8 μg/ml; at 40.5°C the dissymmetry (G_{45}/G_{135}) is about 10 in this vicinity. One does not need the photometer to detect this change. At <38°C the mixture appears to be clear for solution light paths of the order of an inch in length; but at >39°C a mixture containing only 5 μg of poly A and 15 μg of L/ml is visibly turbid. Evidently lysozyme forms smaller

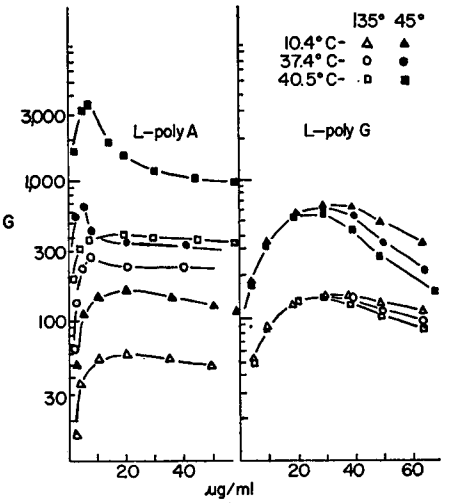

FIGURE 1 Curves of scattered light intensity vs. [homopolymer] at three temperatures. The mixtures contain 15 μg of lysozyme and the weights of poly A or poly G shown on the abscissa scale, per milliliter.

scattering centers with double-stranded, base-stacked poly A than with single-stranded poly A.

No such changes are observed with L-poly G mixtures. Poly G is doubly and triply stranded near these temperatures; it melts far above 60°C, at which temperature the lysozyme itself is altered.

Results like those in Fig. 1 were obtained with poly U, poly I, and poly C. In each case there is a narrow transition region of temperatures for each homopolymer. The intensity and dissymmetry of scattering are strong functions of temperature at homopolymer concentrations where peaks occur at a given temperature. Fig. 2 *a* shows the temperature dependence of scattered light intensity, at 45 and 135° with the transmitted light beam, 15 min after adding L to buffer solutions of the designated homopolymers. After addition [L] = 15 μg/ml and [homopolymer] = 5 μg/ml. The latter concentration was chosen because it is *low enough* to be in the sensitive high scattering region for each homopolymer-lysozyme mixture; other nearby [homopolymers] would suffice. Poly U data are unique in that there is a gradual increase in scattered light intensity well before the abrupt change in dissymmetry. This polynucleotide "melts" at a much lower temperature than do the other three.

Fig. 2 *b* shows data similar to those in Fig. 2 *a*, but for poly G, yeast tRNA, and the complementary pairs poly A-poly U and poly I-poly C. Again [L] = 15 μg/ml and [poly G] = [tRNA] = 5 μg/ml. For the complementary pairs, [poly A] = [poly I] = 6 μg/ml and [poly U] = [poly C] = 6.5 μg/ml. These make the pairs mole to mole in phosphates. In this case the two homopolymers were in-

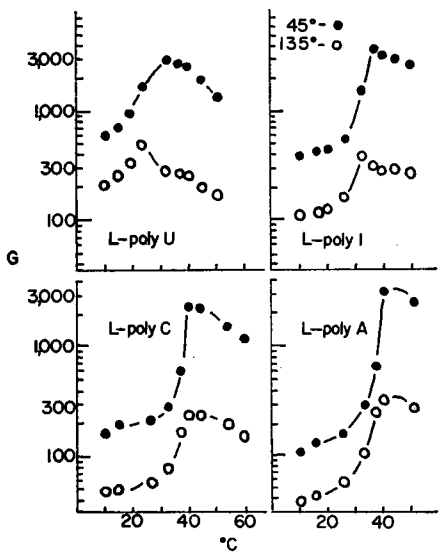

FIGURE 2 *a*

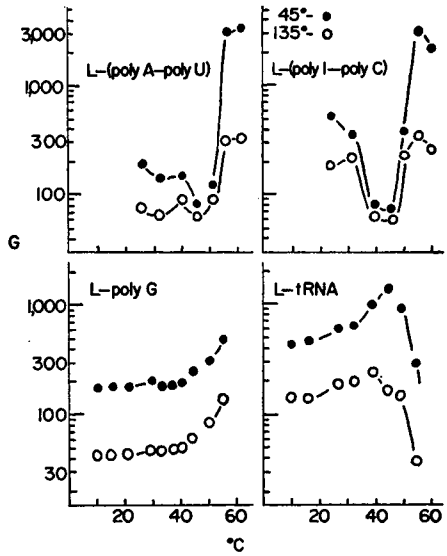

FIGURE 2 *b*

FIGURE 2 *a* Curves of scattered light intensity vs. temperature, 15 min after adding lysozyme. [Homopolymer] = 5 μg/ml.
FIGURE 2 *b* Like Fig. 2 *a*, except that mole ratios in phosphates were maintained for the complementary base pairs (see text).

cubated at room temperature for 5 min, after which the temperature of the mixture was raised (or lowered) to the desired value and maintained at this value during and after lysozyme addition.

Observe the rich variety of scattered light intensity and dissymmetry changes for the curves of Figs. 2 *a* and 2 *b*. Evidently there are configuration changes that are not readily detected by conventional UV absorption techniques. However, sharp changes in dissymmetry occur in a much narrower temperature region. In Fig. 3 we show $G_{45°}/G_{135°}$ as a function of temperature for all eight polynucleotides. The steep portions of these curves are very close to the regions where scattered light intensity undergoes an abrupt change. All of the mixtures are clear to the left and visibly turbid to the right of the dissymmetry transition region (despite concentrations of only 15 μg/ml for the lysozyme and much less for the polyribonucleotides). The sensitivity of this method depends on getting low enough concentrations of the polynucleotides. One could obtain reasonably good data with a hot water bath and a thermometer.

The data confirm that complementary base pairing did produce the anomalies of our last report (Fig. 4 of reference 1). The order obtained there was I > G ≈ U > C > A; it was closely related to the local chemistry of the bases. However, the results in Fig. 3 give a new order that is clearly related to what has loosely

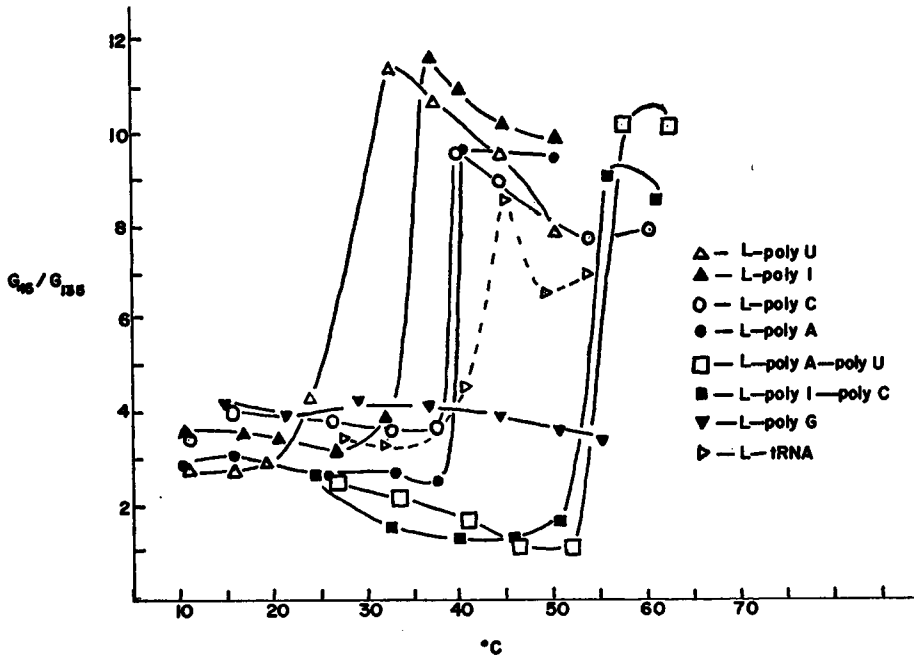

FIGURE 3 Dissymmetry of scattering as a function of temperature. [Polynucleotides] = 5 μg/ml except for the complementary base pairs. With the unaided eye, these mixtures are clear to the left and turbid to the right of the transition regions (see text).

been termed melting. This is

$$\text{poly U} < \text{poly I} < \text{poly C} \approx \text{poly A} < (\text{poly I-poly C}) < (\text{poly A-poly U}) < \text{poly G},$$

as one reads from low to high temperatures. The dotted curve is for a yeast tRNA mixture; it is inserted to show that conformation changes show up in a rather definite region. We think that the technique would perhaps distinguish the separate tRNAs. Note that the poly G curve is flat and relatively low, with $G_{45°}/G_{135°}$ equal to about 4 over the entire range covered. This homopolymer dissociates to single strands far above 60°C in a region where the lysozyme itself would change configuration.

It is true that scattering centers have to be large when dissymmetries are over 3 (1, 3). However, interference optics shows that a relatively small increase in dimensions produces a relatively large increase in dissymmetry when the dissymmetry is over 3 (for any shape) (3). That is what we see in Fig. 3. Low density scattering centers have gone from large to super-large.

In the experiments described so far lysozyme and polynucleotides were brought to the same temperature and then combined. Hence, configuration changes of the polynucleotides are produced before contact with the protein; but we could have

added the lysozyme first and then changed the temperature. Once the lysozyme is present, however, the polynucleotides are rigidly locked in the network formed with the protein at high temperatures. The dissymmetry and intensity of scattering remain high when the mixture is cooled. Of course this would not occur if the polynucleotides were cooled before adding the lysozyme. More will be said about this in connection with Fig. 6.

We have still another way of studying the dissociation of Watson-Crick base pairs. Note that the "transition" temperatures of poly A and poly U are below 40°C, while that of the (poly A-poly U) combination is above 50°C. Thus, at 47°C the intensity and dissymmetry of scattering from L-poly U and L-poly A are much greater than they are for the L-(poly A-poly U) mixtures that have equivalent amounts of both polymers (see Figs. 2 *a*, 2 *b*, and 3). Also recall that pancreatic ribonuclease digests poly U (poly A) very rapidly (slowly) (1, 4) and that A-U base pairing impedes the action of this enzyme. We have used these facts in designing the following experiments (series I and II).

(*a*) Mixtures containing both poly A and poly U were brought to temperature T_e as previously described. The volume was 10.2 ml.

(*b*) At time $t = 0$, 1.2 ml of buffer containing 0.143 μg of pancreatic ribonuclease/ml was added at temperature T_e.

(*c*) Series I, at $t = 60$ s. Series II, at $t = 180$ s. The scattering cell was placed in an ice bath for up to $\Delta t = 30$ s and then put in a constant temperature bath at 47°C for up to 50-Δt s. After these steps the cell was put back in the photometer and maintained at 47°C.

(*d*) Series I, at $t = 140$ s. Series II, at $t = 260$ s. 1.8 ml of buffer containing 100 μg of L/ml was added, so that the final concentrations in 13.2 ml were [poly U] = 5.45 μg/ml, [poly A] = 5.92 μg/ml [L] = 13.65 μg/ml and [R] = 0.013 μg/ml.

(*e*) Series I, at $t = 300$ s. Series II, at $t = 300$ s. Read the scattered light intensity at 45 and 135°.

Results are shown in Fig. 4. For curves I (II) the R was present for 1 (3) min at the elevated temperature. At this small [R], digestion of poly A is negligible at all of these temperatures; however, within 3 min the given amount of poly U is completely digested at all of these temperatures if no poly A is present. The curves show the scattering at 47°C after lysozyme addition. The abscissa is the temperature at which the ribonuclease was added.

If poly U had been completely hydrolyzed, one would obtain the very high scattering and dissymmetry characteristic of the L-poly A interaction at 47°C. On the other hand, if no poly U had been hydrolyzed, one would get the low scattering and dissymmetry characteristic of the L-(poly A-poly U) interaction at 47°C. Curves I and II in the figure are intermediate to these extremes; in exp. I there is more poly U left at L addition than there is in exp. II. We know that digestion of poly U by R is a smoothly increasing function of temperature in this region. Fig. 4 shows that it is not when poly A is present; there is a rather sharp curvature

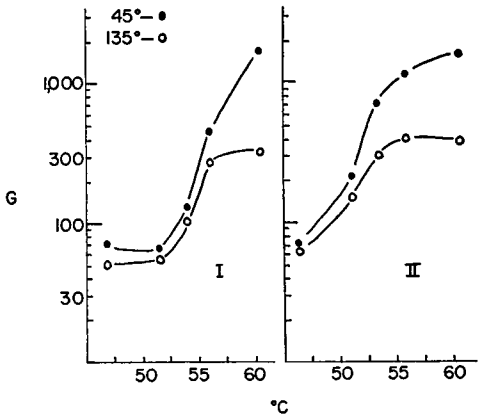

FIGURE 4 The scattered light intensity of L-(poly A-poly U) mixtures at 47°C as a function of the temperature at which digestion by ribonuclease commences. In series I (II) ribonuclease was present for 140 (260) s before lysozyme addition (see text). Breaks in both curves appear where the poly A and poly U separate (poly U digested faster as random coil).

change in the neighborhood of the transition temperature. This can only be a result of R depolymerizing free poly U strands above the transition temperature more rapidly than it does poly A-bound poly U strands below this temperature.

None of the experiments so far described could be carried out with R because (*a*) poly U and poly C are rapidly digested, and (*b*) the scattering from R-homopolymer mixtures decreases with increasing temperature (just the opposite of the behavior with L). Poly I and poly G are not digested by R. Fig. 5 shows the results of adding R to buffered solutions of these homopolymers at three temperatures; it is analogous to Fig. 1 except that R is substituted for L. The dissymmetry of scattering changes little with temperature (it did with L). The R-poly G interaction is practically as unresponsive to temperature as was the L-poly G interaction; however, the scattered light intensity from R-poly I mixtures decreases with temperature (the opposite of what happens with L-poly I mixtures).

Another major difference is apparent from the interactions of the two proteins with poly I. In separate experiments R and L were added to buffer solutions, in which poly I was adjusted to the value where scattered light intensity is at its highest level when the mixtures contain 15 μg of either protein per milliliter. Note from Fig. 3 that the transition temperature of poly I is well below 37°C. R (or L) was added to buffer solutions of poly I at 15°C (or 37°C). The scattering was measured for up to 15 min at which time the temperature was rapidly (within 2 min) brought back to 37°C (or 15°C) (with a water bath at 47°C or an ice bath). The scattering was then measured for another 20 min or so. Results are shown in Fig. 6. L-poly I scattering centers become much larger (dissymmetry increases) when the temperature is rapidly raised and remain large when temperature is rapidly lowered.

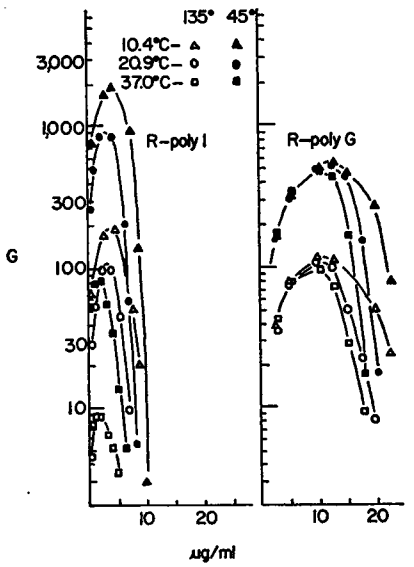

FIGURE 5

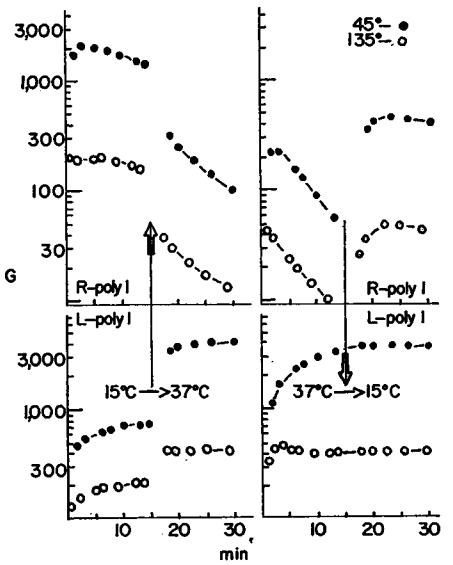

FIGURE 6

FIGURE 5 These curves are analogous to those in Fig. 1, but with R substituted for L. Note the reversed temperature response with poly I (melts near 30°C) and the lack of response with poly G (melts far above 60°C).

FIGURE 6 Effects on R-poly I and L-poly I scattering centers, produced by rapidly changing temperature from 15 to 37°C (or from 37 to 15°C). The transition region for poly I is in between these temperatures (see text).

However, the number per unit volume of R-poly I centers (not the size, because dissymmetry changes little) decreases when the temperature is rapidly raised, but increases again when the temperature is rapidly lowered. It is clear that L-poly I binding prevents the reestablishment of the tighter poly I configuration at the lower temperature. Evidently R-poly I binding cannot do this.

Note that for R data the scattered light intensity decreases rather rapidly with time, at 37°C. This instability of the R-poly I centers is not a consequence of enzymic action of R toward poly I, as one might falsely conclude. If L is added at any time after this process commences, the intense scattering caused by L-poly I centers quickly appears. In earlier work (4) we showed that the scattering from R-poly A mixtures also decreases with time. However, if one adds L too long after this process starts there is no increase in scattering, because there are no long poly A chains left to form the L-poly A centers. R has digested the poly A.

In Table II we have summarized some of the experiments with L involving rapid temperature changes and dissymmetry. Note that the large dissymmetry changes are only observed when the interval defined by the change in temperature includes the transition temperature for the particular polynucleotide (see Fig. 3 and compare with this table). Results are also shown when Watson-Crick pairing occurs.

TABLE II

RAPID TEMPERATURE CHANGES

L-H_i	$\frac{[H_i]}{[L]}$	Dissymmetry $\left(\frac{G_{45}}{G_{135}}\right)$			
		15°C ⇒ 37°C		37°C ⇒ 15°C	
L-poly U	$\left(\frac{15\ \mu g/ml}{15\ \mu g/ml}\right)$	3.1	10.2	10.4	11.4
L-poly I	$\left(\frac{10\ \mu g/ml}{15\ \mu g/ml}\right)$	3.5	9.9	8.8	8.9
L-poly C	$\left(\frac{7.5\ \mu g/ml}{15\ \mu g/ml}\right)$	4.1	4.2	4.0	4.1
L-poly A	$\left(\frac{5\ \mu g/ml}{15\ \mu g/ml}\right)$	3.2	3.6	3.9	3.9
L-poly G	$\left(\frac{30\ \mu g/ml}{15\ \mu g/ml}\right)$	4.4	4.5	4.2	4.2
L-(H_i + H_j)	$\frac{[H_i] + [H_j]}{[L]}$	Dissymmetry $\left(\frac{G_{45}}{G_{135}}\right)$			
		20°C ⇒ 62°C		62°C ⇒ 20°C	
L-(poly A + poly U)	$\left(\frac{6.5\ \mu g/ml + 6.0\ \mu g/ml}{15\ \mu g/ml}\right)$	3.0	10.1	10.1	10.1
		20°C ⇒ 58°C		58°C ⇒ 20°C	
L-(poly I + poly C)	$\left(\frac{6.5\ \mu g/ml + 6.0\ \mu g/ml}{15\ \mu g/ml}\right)$	2.8	8.9	9.1	10.5

The interaction with L at high temperatures (presumably where all polynucleotides are in the form of single random coils) prevents reestablishment of hydrogen bonds when the temperature is lowered. If the temperature were lowered and then the L were added (i.e., the hydrogen bonds were formed first), the dissymmetry would be as low as it would be if the L were added at the lower temperature.

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