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Immunochemistry of Polyribonucleotides. Study of Polyriboinosinic and Polyriboguanylic Acids*

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ABSTRACT: The immunological behavior of polyriboinosinic (poly I) and polyriboguanylic acids (poly G), studied with antiribonucleic acid antibodies, depends on the conformation of these polyribonucleotides and has been employed as a tool for the determination of the number of strands per molecule in solution. In the absence of added electrolytes, and up to a concentration of 10^{-3} M NaCl or 10^{-5} M $MgCl_2$, poly I and poly G appear to precipitate as single-stranded molecules, as judged by comparison with poly U, chosen as the reference for a single-stranded system. At higher salt concentration, the weight of polyribonucleotide precipitable by a given weight of antibody is in direct proportion to the number of strands per molecule. In 5×10^{-4} M $MgCl_2$ and at room temperature, an ordered structure of poly I and poly G is precipitated, immunologically four-stranded. This ordered structure is also present in a 1 M NaCl solution for poly I and 0.25 M NaCl for poly G. At lower salt concentration some multistranded structure persists in solution, to an extent varying with the ionic strength of the solvent. At 45°, poly I precipitates as a single-stranded molecule. Poly-N^7-methylinosinic acid and poly-N^1,N^7-dimethylinosinic acid, which do not form ordered structures, have precipitin curves like those of single-stranded poly U, even in the presence of salts.

The conformation of polyribonucleotides in solution has been studied by various physicochemical methods. In previous investigations, we initiated studies of the role of the conformation of polyribonucleotides in their precipitin reactions with anti-RNA antibodies, isolated by specific precipitation with poly A from the sera of animals hyperimmunized with ribosomes (Barbu and Panijel, 1960; Panijel and Barbu, 1961; Panijel, 1963; Panijel *et al.*, 1963).

These antibodies (to which we refer as NG I antibodies) can precipitate all polyribonucleotides and RNA, but do not cross-react with DNA. These results show that some antigenic determinants are common to all ribonucleic acids and synthetic polyribonucleotides, which cannot serve as immunizing antigens but can act as reactive antigens.

In contrast to most of the antinucleic acid antibodies whose antigenic determinants involve the bases (Tanenbaum and Beiser, 1963; Sela *et al.*, 1964; Seaman *et al.*, 1965; Butler *et al.*, 1965; Plescia *et al.*, 1965) the horse NG I antibodies are capable of precipitating multistranded structures as well as single-stranded structures (Panijel *et al.*, 1966a). However, in the case of multistranded structures the immunological reaction is modified. The amount of antibodies precipitable decreases as compared with the corresponding single-stranded system, while the amount of polynucleotide used as reactive antigen precipitable increases proportionally to the number of strands of the molecule.

This relationship was confirmed in several cases where the number of strands in the molecule was known with certainty, for example, in the case of the

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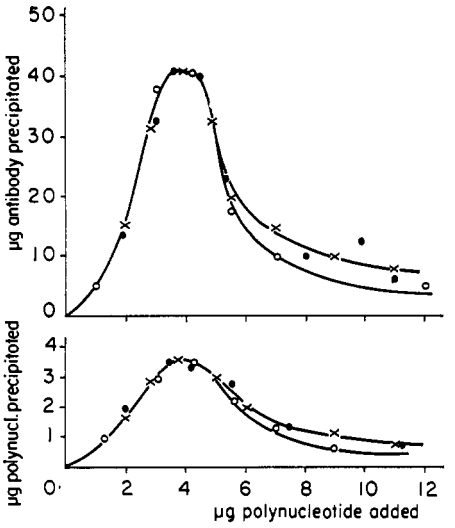

FIGURE 1: Amounts of antibody and antigen precipitated with poly I, poly G, and poly U in the absence of added electrolytes. The amount of antibody was kept constant (110 μg); the polynucleotides were added in increasing amount on a mononucleotidic basis. The reactions were performed at 20° in either 0.005 M Tris-HCl (pH 7.1), EDTA (5×10^{-4} M), or three-times-distilled water adjusted at pH 6.5 with a dilute NaOH solution. In order to reduce nonspecific precipitation of protein, the precipitates were washed with a 0.15 M NaCl–Tris (0.005 M) (pH 7.1) solution. Poly U (×——×), poly I (●——●), and poly G (○——○).

two-stranded complexes, poly[1] (A+U) and poly (C+I), and of the three-stranded complex poly (A+2U) (Souleil *et al.*, 1966). An increase in the amount of nucleotides precipitable was observed similarly under conditions which favor interaction of strands: with poly A at acid pH and with a mixture of poly U and adenylic acid (J. Panijel, unpublished data). On the contrary, this increase of antigen precipitable has never been observed with ordered single-stranded molecules, like poly A or poly C near neutrality (Panijel *et al.*, 1966b). In this report, we have used this particular aspect of the reaction between NG I antibodies and polyribonucleotides as a tool for the determination of the number of strands of poly I and poly G in solution, under various conditions.

It is known that the conformation of poly I depends on salt concentration (Rich, 1958). In 1 M NaCl, at low temperature, several methods, circular dichroism (Brahms and Sadron, 1966), optical rotatory dispersion (Sarkar and Yang, 1965a), and infrared studies (Miles, 1964; Miles and Frazier, 1964; Howard and Miles, 1965), indicate the presence of a highly ordered poly I structure which, according to the X-ray diffraction data (Rich, 1958), is consistent with a tri- or tetra-stranded model. At lower salt concentrations (0.1 M NaCl), poly I is thought to be in a helical single-stranded conformation (Sarkar and Yang, 1965a; Brahms and Sadron, 1966).

Material and Methods

The NG I antibodies were prepared from the euglobulins of sera obtained by hyperimmunization of a horse with 70S ribosomes of *Proteus vulgaris* Boulgakov. The preparation and purification of these antibodies have been described (Panijel *et al.*, 1966b). Briefly, they are isolated by specific precipitation with poly A, dissociated in 1 M $MgCl_2$, chromatographed on Sephadex G 200 in 0.5 M MgCl, and dialyzed successively against 0.15 M NaCl–0.0015 M Tris-HCl (pH 7.4), EDTA (0.002 M), and three-times-distilled water or 0.0015 M Tris (pH 7.1). The salt concentration was adjusted as desired by addition of a concentrated solution. In the absence of buffer, the pH was adjusted to 6.5 before each experiment by addition of dilute NaOH solution.

Poly G (about 8 S) was a generous gift of Dr. M. Grunberg-Manago. Other polyribonucleotides were supplied by Miles Chemical Co. s_{20} of poly U was 5.5. The polyribonucleotides were dissolved in water at a concentration of 1–5 mg/ml. They underwent prolonged dialysis, first against 0.001 M EDTA, then against three-times-distilled water. The stock solutions were kept at −20°.

Immunological reactions were performed in triplicate (except with poly G, available only in small quantity, for which one or two determinations were made according to the concentration). The amount of antibody was held constant (110 μg) and the polyribonucleotides were added in increasing amounts on a mononucleotidic basis. The total volume was 0.2 ml. The antigen and antibody were mixed at room temperature or at 45° and reacted for 4 hr at the same temperature. The specific precipitates were collected by centrifugation, washed three times with 1 ml of 0.15 M NaCl–0.0033 M magnesium acetate–0.005 M Tris-HCl (pH 7), and then dissolved in 0.3 ml of 0.1 N NaOH containing 1% Na_2CO_3. One aliquot was used for estimating the proteins according to the method of Lowry *et al.* (1951). The other served for the estimation of nucleotides using ultraviolet light absorption after 24-hr hydrolysis by 1 N perchloric acid at 37°. The acid-precipitated antibody was removed by centrifugation. Ultraviolet readings were performed at 2480 Å for poly I, 2540 Å for poly G, and 2600 Å for poly U.

Chemical methylation of poly I was carried out using the methods of Michelson and Pochon (1966). Under the conditions employed, methylation using tri-*n*-butylamine and dimethyl sulfate gives only the N^1,N^7-dimethyl polymer. Dimethyl sulfoxide, followed by methyl iodide, gives the N^7-monomethyl polymer.

The immunochemical reactions between NG I antibodies and methylated poly I were performed according to the methods described above. After perchloric acid hydrolysis, ultraviolet readings were performed

[1] Abbreviations used: poly (A+U), the two-stranded complex of the homopolymers poly A and poly U; poly (A+2U), the three-stranded complex of 1 poly A and 2 poly U; poly (C+I), the two-stranded complex of poly C and poly I.

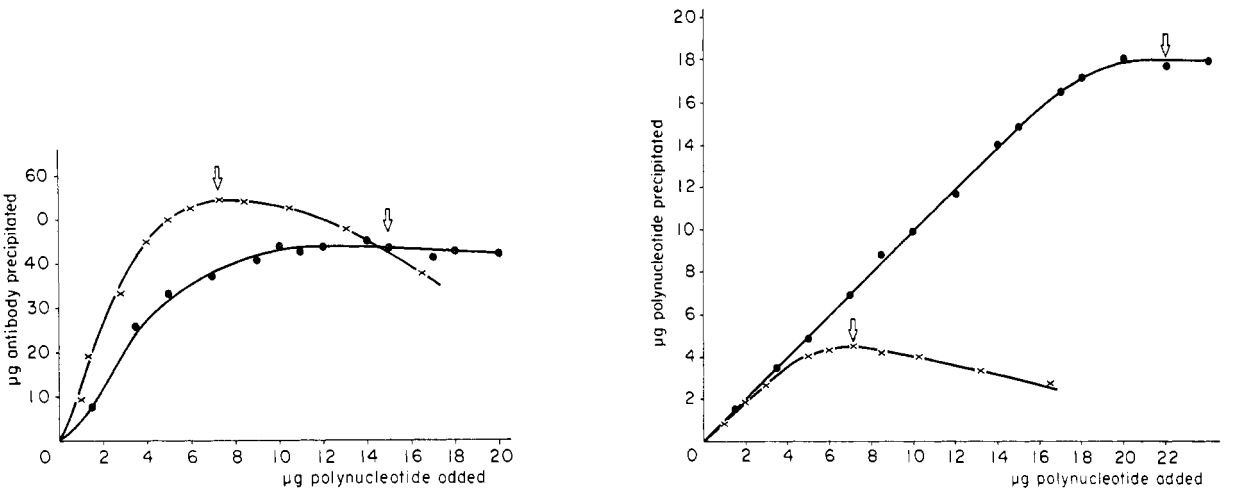

FIGURE 2: Antibody (a) and antigen (b) precipitin curves of poly U and poly I in 5 × 10^{-4} M Mg^{2+}–Tris (0.005 M) (pH 7.1). The arrows indicate the values chosen as representing the equivalence zone and used in the construction of Figures 3 and 4. Poly U (×——×) and poly I (●——●).

at 2500 Å for N^7-methylinosinic acid and at 2620 Å for N^1,N^7-dimethylinosinic acid.

Results

Figure 1 shows that, in the absence of added electrolytes, the curves for the precipitation of either antibody or polynucleotide used as reactive antigen are identical up to the zone of equivalence for poly U, poly I, and poly G. The slight differences appearing in the region of antigen excess can be attributed to differences in the molecular weight of the polynucleotide; using dextrans of varying molecular weight, Kabat (1958) observed a similar phenomenon. These results indicate that the nature of the base is not a quantitative element in the precipitin reaction of these different polyribonucleotides.

At higher ionic strength, the precipitin curves of poly I and poly G differ from those of poly U. As shown in Figure 2 the amount of poly I precipitable exceeds that of poly U (Figure 2b) while the amount of antibody precipitated by poly I decreases as compared with that of poly U (Figure 2a). Quite similar results were obtained with poly G. Our previous study (Panijel *et al.*, 1966a; Souleil *et al.*, 1966) indicated that these immunological characters parallel the precipitation of multistranded molecules of polynucleotidic antigen. Furthermore, there is strong evidence that an increase in salt concentration favors the formation of multistranded structures of poly I or poly G. In contrast, it is well established that poly U is single stranded in the various conditions of our experiments (Lipsett, 1960; Richards *et al.*, 1963; Sarkar and Yang, 1965b). Therefore, the precipitin curves of poly U have been chosen in the present study as immunological reference for a single-stranded molecule.

Precipitin curves of poly U, poly I, and poly G were obtained in media of increasing $MgCl_2$ or NaCl concentration. For each medium, the amount of antibody and polynucleotide precipitable were determined at the zone of equivalence. The ratio of the maximal amounts of poly I to poly U precipitable was calculated, and plotted in Figure 3 as a function of the log of salt concentration; a similar graph was plotted for the maximal amounts of poly G to poly U precipitable. The relationship between the amount of antibody precipitable at the zone of equivalence by poly I and poly U or poly G and poly U (poly U being taken as 100% of precipitation) is shown in Figure 4, again as a function of the log of salt concentration. An increase in the ratio of the maximal amount of antigen precipitated (poly I to poly U or poly G to poly U), accompanied by a decrease in the maximal amount of antibody precipitable, has been interpreted as reflecting only the formation of multistranded structures of poly I or poly G (see below the justification of this working hypothesis).

Study of Poly I and Poly G in $MgCl_2$ Solutions. With no electrolytes added to the reaction medium or up to a concentration of 10^{-5} M $MgCl_2$, the immunological behavior of poly I or poly G is indistinguishable from the single-stranded poly U (Figures 3 and 4). This result suggests that poly I and poly G are single stranded at very low salt concentration and is consistent with the broad profile of the melting curve of poly G in water (Pochon and Michelson, 1965).

With a higher Mg^{2+} concentration, the maximal amount of poly I or poly G precipitable by a given amount of antibody exceeds that of poly U; the ratio followed a sigmoid curve (Figure 3) and reaches a value of 4 for poly I in 5 × 10^{-4} M Mg^{2+}. It remains constant up to a concentration of 8 × 10^{-4} M Mg^{2+}, indicating that the structure precipitated is stable in these conditions. With higher Mg^{2+} concentrations, a spontaneous aggregation of poly I appears during our experimental time.

It has been shown from previous studies (Panijel

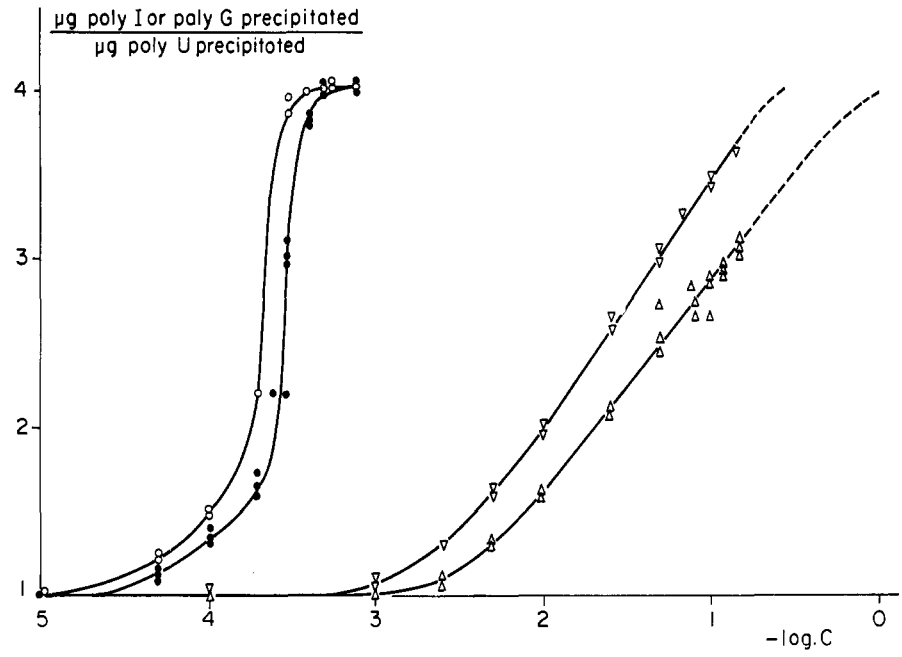

FIGURE 3: Ratios of the maximal amount of poly I (or poly G) precipitated to the maximal amount of poly U precipitated under the same conditions. The experiments were carried out at 20° in Tris (0.005 M, pH 7.1), in increasing concentrations of either $MgCl_2$ or NaCl plus 5×10^{-4} M EDTA. The experiments were repeated in water at pH 6.5 with the same results. All experimentally determined points are indicated on the figure. Poly I in $MgCl_2$ (●——●), poly G in $MgCl_2$ (○——○), poly I in NaCl (△——△), and poly G in NaCl (▽——▽).

et al., 1966a; Souleil *et al.*, 1966) that a direct correlation exists between the number of strands per molecule of polynucleotide used as antigen and the weight of polynucleotide precipitable. Furthermore, poly I, at high salt concentration, is known to be either three or four stranded (Rich, 1958). Therefore, the most evident interpretation of these results is that poly I in 5×10^{-4} M Mg^{2+} assumes a four-stranded structure. The results are similar with poly G, but the four-stranded structure is stable in a 4×10^{-4} M Mg^{2+} solution (Figure 3).

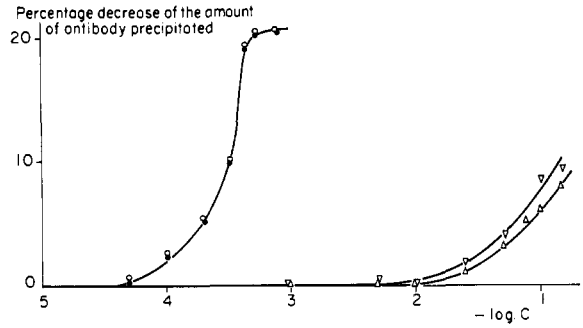

FIGURE 4: Per cent decrease in the amount of antibody precipitated by poly I or poly G at the zone of equivalence, the precipitation of poly U being taken as 100%. The points are the average of three determinations for poly I, two for poly G which was available only in small quantities. The experimentals conditions and symbols used are the same as in Figure 3.

Figure 4 shows that a gradual decrease in the amount of antibody precipitated parallels the precipitation of multistranded molecules of poly I or poly G, reflecting the masking of antigenic sites that we have shown to be a general character of ordered molecules of polyribonucleotides in single- or multistranded conformation (Panijel *et al.*, 1966b; Souleil *et al.*, 1966). The masking of antigenic sites results in a 20% decrease in the maximum amount of antibody precipitable by poly I or poly G as compared with poly U. At low Mg^{2+} concentration, no additional masking can be detected for poly G as compared with poly I, this being probably due to the limits of sensitivity of the antibody dosing technique.

Study of Poly I and Poly G in NaCl Solutions. In solutions of increasing NaCl concentration, the maximal amount of poly I or poly G precipitable exceeds that of poly U in a 10^{-3} M NaCl solution (Figure 3). The profile of the curves of the ratios of the maximal amounts of poly I to poly U precipitable or poly G to poly U precipitable is broader in NaCl than in Mg^{2+}. The immunological behavior of poly I and poly G was only studied up to a 0.15 M NaCl concentration, in view of the inhibition of immunological reactions at high ionic strength. However, it can logically be admitted that the ordered structures of

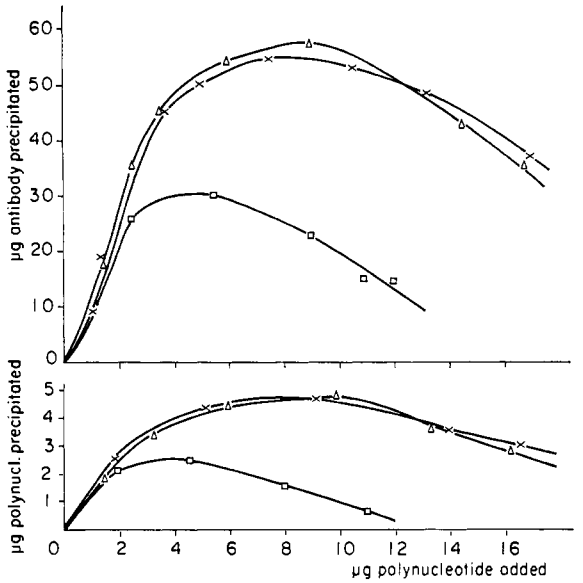

FIGURE 5: Amounts of antibody and antigen precipitated with poly U, poly-N^7-methylinosinic acid, and poly-N^1,N^7-dimethylinosinic acid in 0.005 M Tris–5 × 10^{-4} M $MgCl_2$ at 20°. Compare this figure with Figure 2a,b which shows the precipitin curves of the ordered poly I. Poly U (×——×), poly-N^7-methylinosinic acid (△——△), and poly-N^1,N^7-dimethylinosinic acid (□——□).

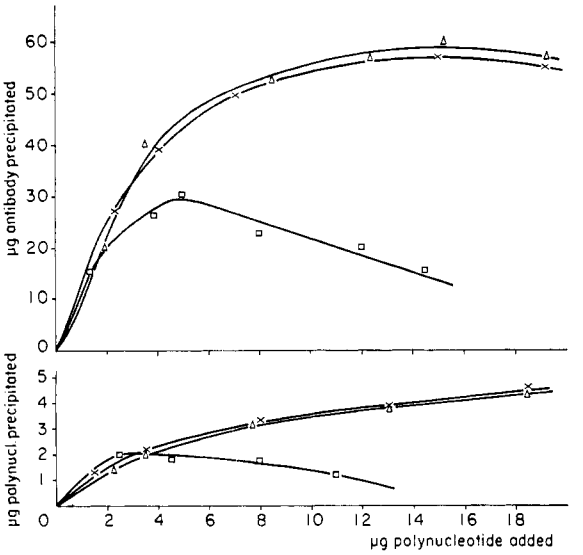

FIGURE 6: Amounts of antigen and antibody precipitated with poly U, poly-N^7-methylinosinic acid, and poly-N^1,N^7-dimethylinosinic acid in 0.005 M Tris, 0.1 M NaCl, and 5 × 10^{-4} M EDTA (pH 7.1). The symbols are those of Figure 5.

poly I or poly G in NaCl do have the same number of strands as the four-stranded molecules precipitated in Mg^{2+} solutions. The extrapolation of the curves of the ratios of the maximal amount of antigen precipitated (poly I to poly U or poly G to poly U) indicates that a 1 M NaCl concentration is necessary for the formation of the four-stranded structure of poly I. A 0.25 M NaCl concentration is only needed with poly G (Figure 3). The precipitation of multistranded molecules of poly I and poly G is accompanied by a decrease in the amount of antibody precipitated, slightly greater in the case of poly G (Figure 4).

Study of Methylated Poly I. In the above experiments, we have assumed that the differences observed in the presence of salts in the precipitin curves of poly I or poly G, on the one hand, and poly U, on the other hand, were due to the precipitation of multistranded molecules of poly I or poly G, resulting in an increasing amount of test antigen precipitated and a decreasing amount of antibody precipitated. In order to show that these immunological characters are effectively linked to the conformation of poly I (or poly G), precipitin reactions have been carried out using as antigen poly I in conditions where its secondary structure is altered. This has been obtained by methylation (leading to the formation of poly-N^7-methylinosinic acid and poly-N^1,N^7-dimethylinosinic acid), or by performing the immunological reactions at 45°.

It is known that methylation of polyribonucleotides deeply affects their conformation (Van Holde *et al.*, 1965). Contrary to poly I, neither poly-N^7-methylinosinic acid nor poly-N^1,N^7-dimethylinosinic acid are capable of forming defined ordered structures (Michelson and Pochon, 1966).

Figures 5 and 6 show the precipitin curves of the methylated poly I and the reference curves of poly U in the same medium. The methylation results in a spectacular modification of the precipitin curves of poly I (compare Figures 5 and 2a,b, which show the precipitation of the ordered poly I in 5 × 10^{-4} M Mg^{2+}). The antigen and antibody precipitin curves of poly-N^7-methylinosinic acid in the presence of salts are identical with those of poly U. With poly-N^1,N^7-dimethylinosinic acid, there is an inhibition of the reaction, the extent of which is independent of the ionic strength of the solvent and can therefore be related to the immunological inhibition usually observed with heterologous antigens.

The maximal amount of antigen precipitated with the two unordered methylated poly I never exceeds that of poly U. Thus, increased amount of poly I precipitable in the presence of salts appears clearly related to the secondary structure of this polyribonucleotide.

Study of Poly I at 45°. The multistranded structure of poly I is probably unstable, as shown by the relatively low temperature of its melting process. At 45° and high salt concentration, the hyperchromicity and optical rotatory dispersion studies of poly I are indicative of a drastic change in the secondary structure, and of a transition to a random coil conformation (Sarkar and Yang, 1965a). Figure 7 shows the antigen precipitin curves of poly I at 45° in 0.1 M NaCl and 5 × 10^{-4}

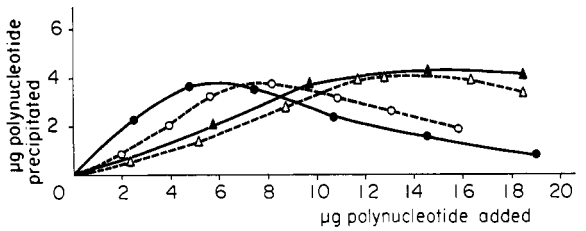

FIGURE 7: Amount of antigen precipitated with poly I at 45° in 0.005 M Tris (pH 7.1) either in 5×10^{-4} M $MgCl_2$ (●——●) or in 0.1 M NaCl (▲——▲). Poly U in 5×10^{-4} M Mg^{2+} (○- - -○) and in 0.1 M NaCl (△- - -△).

M Mg^{2+} (where the four-stranded structure of poly I is observed at room temperature), as well as the reference curves of poly U.

Performing the immunological reaction at 45° results only in a slight decrease of the amount of polynucleotide precipitable with a single-stranded antigen as poly U. In contrast, the amount of poly I precipitable decreases strongly at 45° as compared with the same reaction performed at room temperature (compare Figures 7 and 2b). At 45°, the maximum amount of poly I precipitable is equal to that of poly U, evidencing the dissociation on heating of multistranded molecules of poly I into single-stranded molecules.

Discussion

As in our previous studies (Panijel *et al.*, 1966a,b; Souleil *et al.*, 1966), we used, in the present experiments, purified NG I antibodies produced by treatment with bacterial ribosomes as immunizing antigens. The data reported in this paper underline the role of the conformation of two polyribonucleotides used as specific reactive antigens (poly I and poly G) in their immunological behavior studied with these anti-RNA antibodies. The differences observed in the presence of salts between the precipitin curves of poly I on the one hand, and poly U on the other hand (poly U being chosen as the reference for a single-stranded molecule), disappear when a loss of secondary structure occurs in poly I, either by methylation, or by the transition helix to random coil on heating. The identity of the precipitin curves of poly I, poly G, and poly U when these polyribonucleotides are presumably in single-stranded conformation indicate that the nature of the base (purine or pyrimidine) is not a quantitative element in the immunological reaction, even in the presence of salts. However, an inhibition of the reaction is observed with poly-N^1,N^7-dimethylinosinic acid, raising the question as to whether the imidazole part of the purine ring is involved in an antigenic determinant.

In view of our whole data, it is unlikely that the methyl group in position N^1 is interposed between polynucleotidic antigen and antibody at a binding site; most probably, it is placed in the proximity of the antigenic determinants, thus creating steric hindrance to the approach of the antibodies which results in a partial inhibition of the immunological reaction.

The fact that the reactivity of polyribonucleotides in our immunological system does not seem to involve any base specificity allows the quantitative comparison of their precipitin curves in the same medium. Based on the results obtained with two- and three-stranded complexes (Panijel *et al.*, 1966a; Souleil *et al.*, 1966), a direct correlation can be established between the weight of polyribonucleotide precipitable by a given weight of antibody and the number of strands per molecule of polynucleotide. In the present study, poly U was chosen as the immunological reference for a single-stranded molecule, and the number of strands per molecule of poly I or poly G was determined by the values of the ratio of the maximal amounts of poly I to poly U precipitable or poly G to poly U precipitable.

Only a four-stranded model of poly I or poly G can account for the immunological behavior of these homopolymers at high salt concentration and room temperature. The ordered structure of poly I has been generally considered as three stranded on the basis of the X-ray diffraction data, which however are also consistent with a four-stranded model (Rich, 1958). The results obtained by circular dichroism investigations can also be explained more satisfactorily by a four-stranded model (Brahms and Sadron, 1966). On the other hand, the X-ray diffraction pattern of fibers drawn from solutions of guanylic acid in 0.2 M NaCl at low temperature is compatible with a four-stranded model (Gellert *et al.*, 1962), indicating that this structure is sterically conceivable for the polymer.

In NaCl, the presence of the four-stranded structure of poly I in 1 M concentration is in agreement with the results obtained by several methods, optical rotatory dispersion (Sarkar and Yang, 1965a,b), infrared studies (Miles, 1964), and electron microscopy (Hall, 1959), which, concurrently with the existence of an hypochromie (Rich, 1958) and with the cooperative melting process of poly I heated at high salt concentration (Sarkar and Yang, 1965a), indicate that an ordered structure of this polymer does exist in this condition.

The four-stranded structure of poly G, known to be more stable than poly I, is characterized immunologically in 0.25 M NaCl.

In lower salt concentration, the results of the immunological analysis show that some multistranded structure persists in solution, to an extent varying with the salt concentration, and that poly I and poly G are completely single stranded only at very low salt concentration (10^{-3} M NaCl or 10^{-5} M Mg^{2+}).

A mixture of two-, three-, and four-stranded structures, depending on the salt concentration, could account for our results. However, the relatively broad profile of the melting curve of poly I in 0.1 M NaCl (Sarkar and Yang, 1965a) and the fact that the various physicochemical studies indicate a loss of secondary structure are more compatible with a partial presence of the ordered structure, four-stranded molecules being progressively dissociated into single-stranded molecules,

when the salt concentration is decreased (or the temperature increased).

This immunological method of analysis of polyribonucleotides cannot give any information on the arrangement of the strands in the ordered structure of either poly I or poly G. This structure can be assumed as helical on the basis of the X-ray diffraction data, which represent the direct approach to the study of these molecules.

However, the study of polyribonucleotides in solution and the determination of the conformational changes induced by varying the solvent conditions make it necessary to employ indirect methods. Among these, the estimation of the weight of polyribonucleotide precipitable by a given weight of anti-RNA antibodies appears as a simple and useful means of determining in solution the number of strands of multistranded molecules.

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