References and Notes

1. H. S. Molisch, *Sitzber. Akad. Wiss. Wien.* **120**, 813 (1911); J. Bonner, *Bot. Rev.* **16**, 51 (1950); F. Woods, *ibid.* **26**, 546 (1960); M. Evenari, *Encycl. Plant Physiol.* **16**, 691 (1961).
2. H. Borner, *Bot. Rev.* **26**, 393 (1960).
3. J. T. Curtis and G. Cottam, *Bull. Torrey Bot. Club* **77**, 187 (1950).
4. R. Bernhard, *Bot. Gaz.* **121**, 17 (1959).
5. F. Mergen, *ibid.* **121**, 32 (1959).
6. G. Solomon, *Bot. Rev.* **27**, 422 (1961); S. M. Kupchan, J. R. Knox, J. E. Kelsey, J. A. Saenz Renauld, *Science* **146**, 1685 (1964).
7. I. Lee and M. Monsi, *Bot. Mag. (Tokyo)* **76**, 400 (1963); C. Muller, W. Muller, B. Haines, *Science* **143**, 471 (1964); C. Muller and W. Muller, *ibid.* **144**, 889 (1964); P. Wells, *ibid.*, p. 889.
8. A. Reid, S. Verstrate, C. Wilkei, *Wyoming Range Man.* No. 176 (1963); R. Gray and J. Bonner *Am. J. Botany* **35**, 52 (1948).
9. E. Beals, thesis, Univ. of Wisconsin, Madison, 1961.
10. D. Koller, *Ecology* **38**, 1 (1957).
11. A. Torres, G. Koch, M. Katz, *ibid.* **44**, 414 (1963).
12. B. S. Meyer and D. B. Anderson, *Laboratory Plant Physiology* (Van Nostrand, Princeton, N.J., 1941).
13. J. Harris and R. Gortner, *Am. J. Bot.* **1**, 75 (1914).
14. P. Kramer, *Encycl. Plant Physiol.* **3**, 124 (1956); R. Meyer and J. Gingrich, *Science* **144**, 1463 (1964).
15. L. Dure, *Plant Physiol.* **35**, 919 (1960).

11 March 1966

Immunochemical Characterization of Polyribonucleotides

Abstract. *The degree of organization of polyribonucleotides determines their modalities of reaction with antibodies (NG-I) which are present in serums of animals hyperimmunized with ribosomes. The immunochemical behavior of the highly helical two-stranded complex of polyadenylic acid and polyuridylic acid and the corresponding three-stranded complex of one molecule of polyadenylic acid and two molecules of polyuridylic acid can be determined from examination of the associating and nonassociating mixtures of polyadenylic and polyuridylic acids. The immunochemical characteristics of various forms of polyinosinic acid in solution are described.*

RNA (*1*), DNA (*1*, *2*), and synthetic polynucleotides (*3*) are not immunogenic. However, the presence of antibodies active against denatured DNA has been demonstrated in the serums of patients with systemic lupus erythematosus (*4*). The serums of rabbits immunized with ruptured T-even coliphages similarly contain antibodies reacting with denatured DNA, which are directed, in part, either toward the α-gentiobioside residue of DNA, or toward the α- or β-monoglucosylhydroxymethylcytosine component (*5*).

The antibodies prepared by the procedure of Plescia *et al.* (*5*) had specificities similar to those produced when ruptured bacteriophage was used as the antigen. In addition, various authors (*6*) have investigated the specificity of antibodies resulting from immunization with synthetic agents such that a purine or pyrimidine base was conjugated with a protein antigen. However, even when uridine was conjugated, the resultant antibodies reacted indifferently with heat-denatured DNA and RNA, but not with double-stranded DNA or native RNA.

Antibodies from the serums of animals hyperimmunized with ribosomes (*7*) differ markedly from the above-mentioned antibodies. In the first place, they are produced under very different immunogenic conditions. Second, certain of these antibodies—to which we refer as "NG-I" antibodies, and which we have isolated and purified from horse serums by two or three specific precipitations with polyA (*8*)—precipitate native RNA (soluble, ribosomal, and viral) and various polyribonucleotides, although they still remain inactive with respect to native and denatured DNA.

Furthermore, the immune reaction with NG-I is not inhibited by purine or pyrimidine bases, or by ribo- or deoxyribonucleosides, or by mono-, di-, or trinucleotides (*7*). The foregoing results signify that at least a part of the antigenic determinant is common to all ribonucleic acids and synthetic polyribonucleotides.

We now report on how the extent of organization of polyribonucleotide determines its immunochemical behavior with relation to these NG-I antibodies. First of all, we studied the precipitation near neutrality of polyU and of polyA (*8*) with NG-I antibodies in the absence of electrolytes as well as in the presence of $5 \times 10^{-4}M$ Mg^{++} (Fig. 1, *a* and *b*). Since polyU is randomly coiled under normal conditions, as shown by the failure of the physicochemical investigations to obtain any indication of ordered structure (*9*), the precipitation of antibody and of antigen can be taken as the norms of the immunochemical reaction of an unordered polyribonucleotide (*10*).

The curves of precipitation of polyA near neutrality are identical, up to the zone of equivalence, to those plotted for polyU. This shows (i) that the nature of the base is not a quantitative determinant in the reaction, and (ii) that, under these conditions of ionic strength and *p*H, polyA does not have intermittent ordered regions, which would have decreased the amount of antibody precipitated (*11*).

In the presence of excess antigen, inhibition of the precipitation of polyA is less than that noted under the same conditions for polyU. This is probably related to the lower molecular weight of polyU as compared with that of polyA (*12*). Kabat (*13*) observed the same phenomenon in the case of dextrans of varying molecular weight.

The following experiments show that the immunochemical behavior of highly helical polyribonucleotide complexes such as poly(A+U) and poly(A+2U) (*14*) differs greatly from that of unorganized homopolymers. The immunochemical behavior of these complexes can only be appreciated with reference to the behavior of a corresponding non-

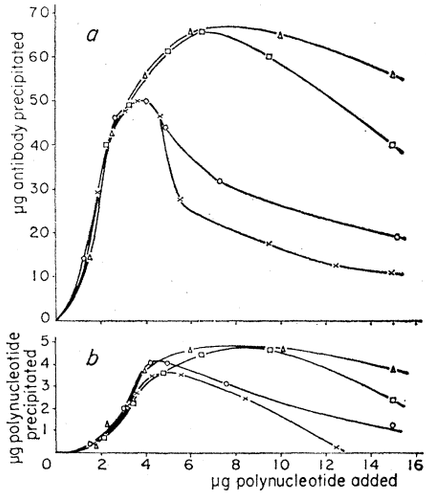

Fig. 1. Amounts of antibody (*a*) and polynucleotide (*b*) precipitated by polyU and polyA ("neutral form"). The polyribonucleotides (Miles) were dialyzed against $3 \times 10^{-3}M$ EDTA and then thrice-distilled water. Stock solutions of antibodies and polynucleotides, stored at −20°C in the absence of electrolytes, were diluted in thrice-distilled water and adjusted to *p*H 7.4, and $5 \times 10^{-4}M$ Mg^{++} when indicated. The polyribonucleotides were added in ever-increasing amounts on a mononucleotidic basis, the amount of antibody being kept constant (110 µg). The specific precipitates were collected, washed three times in ten volumes of $0.1M$ NaCl, $0.0033M$ Mg^{++} acetate, $0.0017M$ tris buffer, *p*H 7.4, and then dissolved in 0.3 ml of $0.1N$ NaOH containing 1 percent Na_2CO_3. One portion of the specific precipitate was used for estimating the proteins (*18*). The other served for the estimation of nucleotides: optical density measurements were made at 260 mµ for polyU and at 257 mµ for polyA, after hydrolysis by $1N$ perchloric acid. ×———×, PolyU in absence of electrolytes; ○———○, polyA in absence of electrolytes; □———□, polyU in $5 \times 10^{-4}M$ Mg^{++}; △———△, polyA in $5 \times 10^{-4}M$ Mg^{++}.

associating mixture of polyribonucleotides in the same medium. However, experimental curves of nonassociating mixtures of polyA and polyU cannot be obtained in the presence of Mg^{++}, since the presence of salts always results in the formation of the poly(A+U) or the poly(A +2U) complex. We therefore constructed curves calculated from the average of the individual values in $5 \times 10^{-4}M$ Mg^{++} for the polyribonucleotides of the mixture, the ratio used being taken into account, that is, U/A=1/1 for poly(A+U) complex, and U/A = 2/1 for poly(A + 2U). Since the experimental and calculated curves are identical in the absence of electrolytes (Figs. 2 and 3) (that is, in a medium where no complexes are formed spontaneously)—an indication that there is no reciprocal inhibition of precipitation of polynucleotides—the calculated curves represent the precipitation in $5 \times 10^{-4}M$ Mg^{++} expected for a theoretical nonassociating mixture of unordered polynucleotides. The results were as follows:

1) With respect to the amount of antibody precipitated in $5 \times 10^{-4}M$ Mg^{++} (Fig. 2), the experimental values were less (by 20 percent in the zone of equivalence) than the theoretical values, for both mixtures of polyA plus polyU and of polyA plus 2 polyU. Up to the zone of equivalence, the curves of precipitation for both complexes were identical, but inhibition of precipitation in excess antigen is less marked in the case of poly(A + 2U).

Hence, the formation of the complexes brings about a masking of antigenic sites and a consequent reduction in the amount of antibody precipitated as compared with a nonassociating mixture. However, the addition of a supplementary strand, that is, the formation of the poly(A+2U) complex, does not increase this masking. This is consistent with the hypothesis that the new strand of polyU is inserted in the wide helical groove of the poly(A+U) complex without structurally reorganizing the double-stranded molecule (*15*) and, therefore, without modifying the distribution of antigenic sites.

2) With respect to the amount of antigen precipitated in $5 \times 10^{-4}M$ Mg^{++} (Fig. 3), the maximum value appearing in the theoretical curve (the curve for a nonassociating mixture polyA + polyU or polyA + 2 polyU) corresponds to the amount precipitated by a single strand. On the other hand, the maximum amount of antigen precipitated with the double-stranded complex is twice as great as the maximum amount precipitated by a single-stranded polynucleotide. Similarly, with the triple-stranded complex, the maximum value is three times the maximum amount precipitated by the single-stranded molecule.

There is, therefore, an evident relation between the maximum amount of nucleotidic material precipitated and the number of strands constituting the polynucleotide taking part in the reaction. We have used this relation in the study of polyI (Fig. 4), where the number of strands in solution has not yet been clearly established (*16*). At *p*H 7.4, in the absence of electrolytes, the maximum amount of polyI precipitated corresponds to that precipitated with a single-stranded polynucleotide, as compared with polyU and the "neutral form" of polyA. However, at *p*H 6.5 in the absence of electrolytes, the amount of precipitation doubles, an indication of the probable formation of a double-stranded molecule. In 0.05*M* and 0.09*M* NaCl, at *p*H 7.4, the results similarly point toward a double-stranded structure (*17*). PolyI, in 0.14*M* NaCl, assumes a triple-stranded form, this being the one most often cited (*16*). Finally, in $5 \times 10^{-4}M$ Mg^{++}, the amount of precipitate formed indicates the presence of a four-stranded molecule.

Certain principles of immunochemical analysis of polyribonucleotides with NG-I antibodies can be drawn from these results. In the first place, the degree of organization, and not the nature of the base, determines the immunochemical reactivity of polyribonucleotides. Second, by considering the polyribonucleotide molecule as a structural unit along which the antigenic sites are

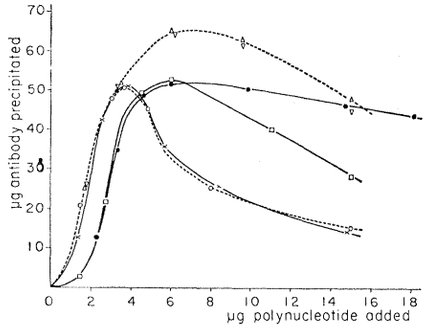

Fig. 2. Comparison of the amount of antibody precipitated in mixtures of polyA and polyU in absence of electrolytes and in $5 \times 10^{-4}M$ Mg^{++}. The theoretical curves were calculated from the individual precipitation of each constituent polynucleotide, in the corresponding medium. PolyA and polyU were mixed at *p*H 7.4 in $5 \times 10^{-4}M$ Mg^{++} in the following ratios of concentration: polyA to polyU equal to 1 : 1, for the formation of the poly (A + U) complex, and polyA to polyU equal to 1 : 2, for the formation of the poly(A + 2U) complex. The complexes were used after 90 minutes in the case of the double-strand (25 percent final hypochromia at 260 mµ), and 120 minutes for the triple-strand (34 percent final hypochromia at 260 mµ). ×———×, Observed values of the polyA + polyU mixture in absence of electrolytes; ○———○, calculated values of a theoretical mixture of polyA + polyU in absence of electrolytes; □———□, poly(A + U) complex in $5 \times 10^{-4}M$ Mg^{++}; △———△, calculated values of a theoretical mixture of polyA + polyU in $5 \times 10^{-4}M$ Mg^{++}; ●———●, poly(A + 2U) complex in $5 \times 10^{-4}M$ Mg^{++}; ▽———▽, calculated values of a theoretical mixture of polyA + 2 polyU in $5 \times 10^{-4}M$ Mg^{++}.

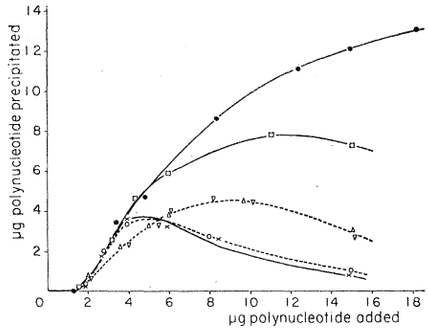

Fig. 3. Comparison of the amount of polynucleotide precipitated (on a mononucleotidic basis) in the case of mixtures of polyU and polyA at molar ratios of 1 : 1 and 2 : 1, in the absence of electrolytes and in $5 \times 10^{-4}M$ Mg^{++}. All symbols and theoretical curves as in Fig. 2.

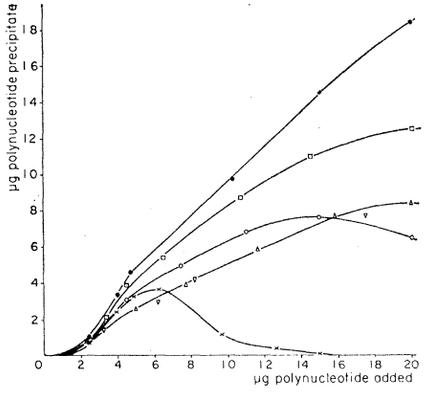

Fig. 4. Amount of polynucleotide precipitated in the case of polyI. The optical density measurements were made at 248 mµ. ×———×, PolyI in absence of electrolytes at *p*H 7.4; ○———○, in absence of electrolytes at *p*H 6.5; ▽———▽, in 0.05*M* NaCl; △———△, in 0.09*M* NaCl; □———□, in 0.14*M* NaCl; ●———●, in $5 \times 10^{-4}M$ Mg^{++}.

disposed, it is easy to see that the amount of specifically precipitated nucleotidic material is proportional to the number of strands involved in the reaction. With this observation in view, the number of strands in a molecule can be determined immunochemically if reference values corresponding to an unordered single-stranded molecule are available.

Finally, the organization of polyribonucleotides determines the degree of masking of antigenic sites. The antigenic sites are probably located along the polyribosephosphate backbone. This statement is supported by the facts that, on the one hand, the nature of the base is not a quantitatively determining factor in the reaction, and, on the other hand, the NG-I antibodies can still precipitate two-, three-, and even four-stranded molecules in which the bases are hidden in the interior of the molecule. The establishment of a helical structure would create, at regular intervals, a steric hindrance to the approach of antibodies, and cause masking of antigenic sites which results in a decrease in the amount of antibody precipitated.

JACQUES PANIJEL
CLAUDE SOULEIL
PHILIPPE CAYEUX

Service des Streptocoques, Institut Pasteur, 25 rue du Docteur Roux, Paris 15e, France

References and Notes

1. G. Pasternak, F. Hoffman, H. Bielka, L. Venker, *Pathol. Microbiol.* **23**, 392 (1960).
2. J. S. Colter, *Nature* **190**, 550 (1961); S. Yachnin, *J. Immunol.* **91**, 529 (1963); R. H. Wilson, V. Jurevics, A. C. Schram, *Fed. Proc.* **24**, 185 (1965).
3. S. Yachnin, *Nature* **195**, 1319 (1962).
4. M. Seligman, *Compt. Rend.* **245**, 243 (1957); H. R. G. Deicher, H. R. Holman, H. G. Kunkel, *J. Exp. Med.* **109**, 97 (1959); D. Stollar and L. Levine, *J. Immunol.* **87**, 477 (1961); ———, H. I. Lehrer, H. Van Vunakis, *Proc. Nat. Acad. Sci. U.S.* **48**, 874 (1962).
5. L. Levine, W. T. Murakami, H. Van Vunakis, L. Grossman, *Proc. Nat. Acad. Sci. U. S.* **46**, 1038 (1960); W. T. Murakami, H. Van Vunakis, H. I. Lehrer, L. Levine, *J. Immunol.* **89**, 116 (1962); E. E. Townsend, H. Van Vunakis, L. Levine, *Biochemistry* **4**, 943 (1965); O. J. Plescia, W. Braun, N. C. Palczuk, *Proc. Nat. Acad. Sci. U.S.* **52**, 279 (1964).
6. V. P. Butler, S. M. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, A. Bendich, *Proc. Nat. Acad. Sci. U.S.* **48**, 1597 (1962); S. W. Tanenbaum and S. M. Beiser, *ibid.* **49**, 662 (1963); M. Sela, H. Ungar-Waron, Y. Schechter, *ibid.* **52**, 285 (1964); V. P. Butler, S. W. Tanenbaum, S. M. Beiser, *J. Exp. Med.* **121**, 19 (1965).
7. E. Barbu and J. Panijel, *Compt. Rend.* **250**, 1382 (1960); J. Panijel, *ibid.* **256**, 4540 (1963); ———, *ibid.* **257**, 3064 (1963).
8. Abbreviations: polyA, polyriboadenylic acid; polyU, polyribourydilic acid; poly(A + U), the two-stranded complex of the homopolymers polyA and polyU; poly(A + 2U), three-stranded complex of 1 polyA and 2 polyU; polyI, polyriboinosinic acid; EDTA, ethylenediaminetetraacetate.
9. E. G. Richards, P. Flessel, J. R. Fresco, *Biopolymers* **1**, 431 (1963); P. K. Sarkar and J. T. Yang, *J. Biol. Chem.* **240**, 2088 (1965); M. N. Lipsett, *Proc. Nat. Acad. Sci. U.S.* **46**, 443 (1960); J. Brahms, *J. Am. Chem. Soc.* **85**, 3298 (1963).
10. Because of the low salt concentration, we attribute the increase in precipitated antibody in $5 \times 10^{-4}M$ Mg^{++} to the overall state of the antigen and the neutralization of its negative phosphate charges rather than to an influence of the salt on the interaction of specific sites of antigen and antibody.
11. O. N. Holcomb and I. Tinoco, Jr., *Biopolymers* **3**, 121 (1965); J. Panijel, C. Souleil, P. Cayeux, Information Exchange Group 7, No. 118; ———, *Biochim. Biophys. Acta*, in press.
12. Sedimentation coefficients, determined in $0.1M$ NaCl, $0.01M$ tris buffer, *p*H 7.4, were $S_{20} = 5.5$ for polyU and $S_{20} = 12.3$ for polyA.
13. E. A. Kabat, *Arch. Biochem. Biophys.* **78**, 306 (1958).
14. A. Rich and D. Davies, *J. Am. Chem. Soc.* **78**, 3548 (1956); G. Felsenfeld and A. Rich, *Biochim. Biophys. Acta* **26**, 457 (1957); G. Felsenfeld, *ibid.* **29**, 133 (1958); R. F. Steiner and R. F. Beers, *Polynucleotides* (Elsevier, Amsterdam, 1961); J. R. Fresco, in *Informational Macromolecules*, H. I. Vogel, V. Bryson, J. O. Lampen, Eds. (Academic Press, New York, 1963), p. 146.
15. G. Felsenfeld, D. Davies, A. Rich, *J. Am. Chem. Soc.* **79**, 2023 (1957).
16. A. Rich, *Biochim. Biophys. Acta* **29**, 502 (1958); P. Doty, H. Boedtker, J. R. Fresco, R. Haselkorn, M. Litt, *Proc. Nat. Acad. Sci. U.S.* **45**, 482 (1959); G. Giannoni and A. Rich, *Biopolymers* **2**, 399 (1964).
17. The observed differences between the curves of reactions at *p*H 6.5 in the absence of electrolytes, and in $0.05M$ or $0.09M$ NaCl, are due to the influence of salt on the immunological reaction.
18. O. H. Lowry, N. T. Rosenbrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
19. Supported by D.G.R.S.T. grant (Molecular Biology).

24 January 1966

Beta-Glucuronidase Activity in Serum Increased by Coronary-Artery Atherosclerosis

Abstract. *Increase in activity of beta-glucuronidase in serum has been demonstrated in patients having clinically evident coronary-artery atherosclerosis. This fact, yielded by the new, more specific method of Fishman, could not be elicited by the traditional method.*

Use of Fishman's new and more specific method for the determination of beta-glucuronidase activity in serum (*1*) has enabled demonstration of markedly increased activity in patients having coronary-artery atherosclerosis. The new method employs sixfold increase of the phenolphthaleinglucuronide substrate and reduces the time of incubation from 20 to 4 hours; it gives values roughly twice as high as those obtained by his previous method (*2*), and the new values correspond closely with values obtained from serum from which inhibitors have been removed by dialysis.

Our study dealt with 49 patients of whom each had either classical symptoms of angina pectoris or a documented history of coronary-artery occlusion with myocardial infarction (in patients in whom the clinical episode had occurred months or years previously, so that one may assume that their increased beta-glucuronidase activities in serum did not result from ischemic necrosis of cardiac muscle). None of the patients showed clinical or laboratory evidence of diabetes mellitus, liver disease, or pregnancy, any of which increases beta-glucuronidase activity.

The controls selected were patients either attending the hospital diagnostic clinic or awaiting elective surgery such as herniorrhaphy; all were free of clinically apparent cardiovascular diseases or other overt illness, and matched the coronary-atherosclerosis patients as closely as possible as to age, sex, and race.

The 49 test patients showed an average activity of 2140 units, versus 1270 units for the 67 controls ($P < .001$). Eighty-five percent of the controls gave values below 2000 units, whereas 58 percent of the test patients ranged above 2000 units; only one control exceeded 2500 units, whereas 37 percent of the test patients ranged between 2500 and 5310 units. When the results for the two groups were compared by age, sex, or race, activity was always significantly higher for the coronary patients than for the controls (*3*).

Others (*4*) were unable to demonstrate increased activity in patients having coronary-artery disease by the traditional method of Fishman, Springer, and Brunetti (*2*), which we also used on each of the specimens of serum studied by the new Fishman procedure. The less specific method showed no significant differences in mean enzyme activity between the test patients and the controls. A fluorometric procedure has shown a suggestive increase in female but not in male patients (*5*).

Several workers using the less specific method have found increased