Immune Response to the Collagen-like Synthetic Ordered Polypeptide (L-Pro-Gly-L-Pro)$_n$†

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ABSTRACT: Specific antibodies to the periodic collagen-like polypeptide (L-Pro-Gly-L-Pro)$_n$ were obtained in several species by immunization with the free polymer or with covalent conjugates of the polymer with a carrier protein. Immunization with the conjugate (Pro-Gly-Pro)$_n$–ovalbumin resulted in higher anti-(Pro-Gly-Pro)$_n$-antibody titers than those obtained with the free polymer, although the specificity of these antibodies was in both cases directed exclusively against the conformational determinants in the periodic polymer. Antibodies to the ordered polymer were shown to be specific to the unique collagen-like conformation of the immunogen as they cross-react with other collagen-like polyhexapeptides and to a lesser extent with the random copolymer (L-Pro^{66},Gly^{34})$_n$ which is not collagen-like. The extent of cross-reactivity of these antibodies with different polymers tested depends both on the size and conformation of the polymer. Collagenase digestion of (Pro-Gly-Pro)$_n$ abolishes its binding to the antibodies. The tripeptide Pro-Gly-Pro, which is the building unit of the ordered polymer, does not cross-react with antibodies to the ordered polymer. Antibodies to the tripeptide Pro-Gly-Pro were obtained by immunization with Pro-Gly-Pro–ovalbumin. These antibodies cross-react weakly with the polymer (Pro-Gly-Pro)$_n$. The immune response to (Pro-Gly-Pro)$_n$ was tested at the cellular level by delayed type skin reactions in guinea pigs. The cellular response depends also on the size and conformation of the immunogen. It was demonstrated that the cellular immune response to (Pro-Gly-Pro)$_n$ precedes the humoral response.

The antigenic specificity of proteins is largely controlled by their steric conformation. In protein and polypeptide antigens it is possible to distinguish between sequential and conformation-dependent determinants (Sela *et al.*, 1967; Sela, 1969, 1973). A sequential determinant is defined as one due to an amino acid sequence in a random coil form, and antibodies to such a determinant are expected to react with a peptide of identical, or similar, sequence. On the other hand, a conformational determinant results from the steric conformation of the immunogenic macromolecule, and leads to antibodies that would not necessarily react with peptides derived from that area of the molecule. It seems that antibodies to native proteins are directed mostly against conformational rather than sequential determinants. Schechter *et al.* (1971a,b) investigated synthetic polymers designed on purpose as models for immunogens with sequential and conformational determinants.

The immunogenicity of collagen is an intriguing problem, both from the clinical and structural point of view. Collagen was considered for many years to be a very poor immunogen. With the development of sensitive immunological techniques for detection of antibodies it became apparent that collagen can be immunogenic. Immunological properties of collagen were summarized in several review articles (O'Dell, 1967; Kirrane and Glynn, 1968; Benjamini *et al.*, 1972). Arnon and Sela (1960) investigated the immunological properties of gelatin to which various peptides were attached, and Kirrane and Robertson (1968) studied the antigenicity of tyrosylated collagen.

Another approach for studying the immunological properties of collagen was to use synthetic models with similar features to those of collagens, and to find out whether there is immunological cross-reaction between such polymers and collagens. The immunological properties of polyproline, polyhydroxyproline, and several linear random copolymers composed of glycine, proline, and/or hydroxyproline were studied (Jasin and Glynn, 1965a,b; Brown and Glynn, 1968). None of the polymers tested cross-reacted with natural collagens. On the other hand, the octapeptide Gly-Pro-Gly-Pro-Pro-Gly-Ala-Lys, synthesized by Kettman *et al.* (1967), was found to cross-react immunologically with collagen.

In order to elucidate the nature of the collagen fold conformation more sophisticated collagen models were synthesized in the last decade by polymerization of tri- and hexapeptides (Carver and Blout, 1967; Piez and Traub, 1971; Segal, 1969). The resulting ordered polypeptides have in common a sequential occurrence of glycine at every third position along the polypeptide chain. The availability of these ordered polymers and the data already accumulated concerning the relationships between their sequence, and physicochemical properties in solution and in the solid state, also permitted the elucidation of the relationship between their chemical nature and their immunological properties. Such polymers are excellent models for studying the role of protein conformation in immunogenicity and antigenic specificity, since their defined sequence makes it possible to follow the immune response against conformational and sequential determinants.

The ordered repeating sequence polymer (L-Pro-Gly-L-Pro)$_n$ was shown to resemble collagen in its three-dimensional structure both in solution (Engel *et al.*, 1966) and in its X-ray diffraction pattern (Traub and Yonath, 1965, 1966). Borek *et al.* (1969) have shown that (Pro-Gly-Pro)$_n$ is immunogenic in guinea pigs and rabbits, and demonstrated, using passive cutaneous anaphylaxis test, a weak cross-reaction with several collagens.

In the present paper we describe the formation and detection of antibodies against (Pro-Gly-Pro)$_n$ in guinea pigs,

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rabbits, and goats, and compare them with antibodies to the sequential determinant Pro-Gly-Pro and to the random copolymer of L-proline and glycine in a residue molar ratio of 2:1, (L-Pro^{66},Gly^{34})$_n$. We have also studied the immunochemical characteristics of the antibodies formed as well as of the purely cellular immune response produced by the ordered periodic polymer, demonstrating the crucial role of the higher order structure of the periodic polymer in defining its immunological properties. The following paper describes the immunological cross-reactions between the synthetic polytripeptide (Pro-Gly-Pro)$_n$ and collagens from several animal species.

Materials and Methods

Synthetic Polymers. The ordered polymer (L-Pro-Gly-L-Pro)$_n$ was synthesized according to the method of Engel *et al.* (1966) by polymerization of the tripeptide L-Pro-Gly-L-Pro in pyridine with tetraethyl pyrophosphite as an initiator. The polymer obtained was chromatographed on a Sephadex G-50 column (6 × 120 cm), and several fractions of different average molecular weights were collected. The first fraction, with the highest molecular weight (6300, determined by Yphantis method (1960)), was used for immunization. The other fractions were used for immunological specificity studies as well as for conjugation with proteins, bacteriophage, or Sepharose. The random copolymer of L-proline and glycine in a molar residue ratio of 2:1, designated as (L-Pro^{66},Gly^{34})$_n$, was prepared as described elsewhere (Borek *et al.*, 1969). This polymer was also chromatographed on a Sephadex G-50 column (6 × 120 cm) and was fractionated into four fractions of different average molecular weights. Poly-L-proline samples of different average molecular weights were synthesized by polymerization of *N*-carboxy-L-proline anhydride (Kurtz *et al.*, 1958), and were obtained from the collection of the Department of Biophysics. The polyhexapeptides (Gly-Ala-Pro-Gly-Pro-Pro)$_n$, (Gly-Pro-Ala-Gly-Pro-Pro)$_n$, (Gly-Ala-Pro-Gly-Pro-Ala)$_n$, and (Gly-Ala-Ala-Gly-Pro-Pro)$_n$ were prepared by polymerization of the respective hexapeptides (Segal, 1969), and were generously provided by Dr. D. Segal.

Protein Conjugates with Synthetic Polymers. Conjugates of ovalbumin (Mann Research Laboratories, N. Y.) or RNase (Sigma Chemicals Co., Mo.) with (Pro-Gly-Pro)$_n$ or (Pro^{66},Gly^{34})$_n$ were prepared in a procedure similar to that described by Goodfriend *et al.* (1964) for the preparation of conjugates of proteins with bradykinin. The protein (100 mg) and the synthetic polymer (200 mg; fractions of an average molecular weight of about 2000 were used) were conjugated by means of water soluble carbodiimide (200 mg of 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide hydrochloride, Ott Chemical Co., Mich.) in a total volume of 1 ml of water. The extent of conjugation was determined by amino acid analysis of the conjugates after hydrolysis. The conjugates (Pro-Gly-Pro$_n$)-ovalbumin and (Pro-Gly-Pro)$_n$-RNase contained 16 and 10 mol of polymer/mol of protein, respectively. The conjugate (Pro^{66},Gly^{24})$_n$-RNase contained 10 mol of polymer/mol of RNase.

Conjugates of the tripeptide Pro-Gly-Pro with ovalbumin or RNase were prepared by reacting the protein (80 mg in 2 ml of water), with *N-tert*-butyloxycarbonyl-Pro-Gly-Pro-succinimide ester (300 mg in 4 ml of mixture of dioxane, dimethylformamide, and water (8:1:2 v/v)) by stirring 24 hr at room temperature. A precipitate formed and the solvents were removed by evaporation *in vacuo*. The precipitate was collected, washed twice with water, and dried. Butyloxycarbonyl groups were removed with trifluoroacetic acid (80% in water) for 30 min at room temperature, following which the solution was diluted in water and dialyzed exhaustively against distilled water. The extent of conjugation was determined by amino acid analysis of the hydrolyzed conjugate. The conjugates Pro-Gly-Pro-ovalbumin and Pro-Gly-Pro-RNase contained 21 and 8 mol of tripeptide/mol of protein, respectively.

Modified Bacteriophage Preparations. (Pro-Gly-Pro)$_n$-RNase-bacteriophage T4 was prepared by conjugation of (Pro-Gly-Pro)$_n$-RNase to T4 phage by using glutaraldehyde as the cross-linking reagent, by a procedure similar to that described by Haimovich *et al.* (1970a) for conjugation of RNase to T4. (Pro-Gly-Pro)$_n$-bacteriophage T4 was prepared by direct conjugation of the polymer (mol wt 2000) to bacteriophage T4 by using 1,3-difluoro-2,4-dinitrobenzene (N_2phF_2; Aldrich Chemicals, Wis.) as a cross-linking reagent, by a procedure similar to that described by Shaltiel *et al.* (1971) for the preparation of conjugates of ampicillin polymers with bacteriophage T4. The (Pro-Gly-Pro)$_n$ (21 mg) was first reacted with N_2phF_2 (1.66 mg) in 0.25 ml of 0.05 M sodium bicarbonate for 7 min at room temperature, and the solution was then added to 0.25 ml of 0.1 M carbonate buffer (pH 9.5) containing 5 × 10^{10} bacteriophages. The mixture was incubated at room temperature overnight. Five milliliters of gelatin-containing phosphate buffer (0.05 M phosphate buffer (pH 6.8) containing 20 μg of gelatin/ml) was added, and the mixture was centrifuged for 1 hr at 20,000*g*; the pellet was resuspended in gelatin-containing buffer. This procedure of centrifugation and resuspension was repeated once more.

Digestion with Collagenase. Collagenase (Worthington Biochemicals Corporation, N. J.) digestion was performed in a procedure similar to that described by Fuchs and Harrington (1970). The polymers (Pro-Gly-Pro)$_n$ or (Pro^{66},Gly^{34})$_n$ were incubated with the enzyme (2% by weight of the polymer) in 0.025 M Tris-HCl buffer (pH 7.4) and 0.01 M $CaCl_2$. The reaction was terminated by dilution of the reaction mixture in a calcium-free Tris-HCl buffer.

Preparation of Immunoadsorbent. (Pro-Gly-Pro)$_n$ was covalently conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala) (Porath *et al.*, 1967). The polymer (700 mg) reacted with 40 g of CNBr-activated Sepharose. Amino acid analysis of the hydrolyzed immunoadsorbent showed that 540 μg of the polymer was covalently conjugated/1 ml of packed adsorbent.

Immunization Procedures. Guinea pigs were immunized as described by Borek *et al.* (1969). The guinea pigs were skin tested for immediate and delayed reactions according to Borek *et al.* (1969). Rabbits were immunized intradermally in multiple sites with the respective immunogen (2 mg/rabbit) emulsified in complete Freund's adjuvant. Injections were repeated after an interval of 1 week. A goat was immunized intradermally in multiple sites with the ordered polymer (Pro-Gly-Pro)$_n$ (5 mg; mol wt 6300) emulsified in complete Freund's adjuvant. Immunization was repeated after an interval of 14 days.

Immunospecific Isolation of Antibodies. Antibodies were isolated from guinea pig, rabbit, or goat antisera against (Pro-Gly-Pro)$_n$ by means of the (Pro-Gly-Pro)$_n$-Sepharose. The adsorption of the antibodies on the immunoadsorbent was performed at 37°. The antibodies were eluted with 1 M acetic acid at the same temperature, and were than dialyzed against phosphate-buffered saline (0.01 M phosphate buffer (pH 7.4) and 0.14 M NaCl) and concentrated by pressure dialysis.

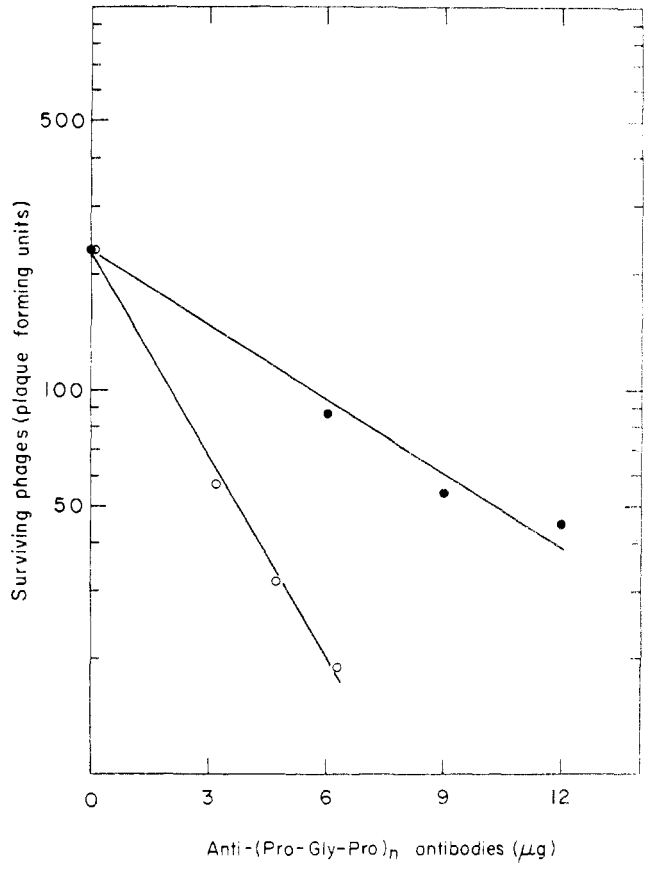

FIGURE 1: Inactivation of (L-Pro-Gly-L-Pro)$_n$–RNase–bacteriophage T4 conjugate by isolated guinea pig anti(Pro-Gly-Pro)$_n$–antibodies (○) and by isolated rabbit anti-(Pro-Gly-Pro)$_n$–antibodies (●).

Quantitative precipitin reactions and inhibition of precipitin reactions were performed as described elsewhere (Fuchs and Sela, 1963). Microhemagglutination assay was done according to the procedure of Herbert (1967), using disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) (Kabat, 1968). Serum dilutions were done in phosphate-buffered saline containing 1% normal rabbit serum. The results were evaluated after an incubation of the plates overnight at room temperature. Inactivation of modified bacteriophage and the inhibition of such inactivation with different inhibitors were performed according to Haimovich *et al.* (1970a,b).

Results

Immunogenicity of (Pro-Gly-Pro)$_n$. It was previously reported (Borek *et al.*, 1969) that the ordered polypeptide (Pro-Gly-Pro)$_n$ was a poor immunogen in rabbits and guinea pigs. Antibody formation could be detected only by passive cutaneous anaphylaxis or by skin reactions (in guinea pigs). Indeed, when we have immunized repeatedly guinea pigs, rabbits, and a goat with (Pro-Gly-Pro)$_n$ (mol wt 6300), the antisera obtained did not give detectable precipitin reactions. Moreover, even when the sensitive assay of the inactivation of chemically modified bacteriophage was applied, the inactivation obtained with undiluted sera from these animals was fairly weak (not exceeding 40% inactivation).

In order to overcome the problem of detecting low titers of antibodies, specific antibodies were purified from various antisera. Rabbit, goat, and guinea pig antibodies to (Pro-Gly-Pro)$_n$ were immunospecifically adsorbed on (Pro-Gly-Pro)$_n$–Sepharose. The adsorbed antibodies were eluted from the immunoadsorbent at 37° with 1 M acetic acid. The amount of purified antibodies obtained corresponded to 7.5 μg/ml, 10 μg/ml, and 7.5 μg/ml of antibodies in rabbit, goat, and guinea pig anti-(Pro-Gly-Pro)$_n$ sera, respectively. The antibodies isolated were tested for their capacity to inactivate the modified bacteriophage (Pro-Gly-Pro)$_n$–RNase T4. Figure 1 describes the inactivation of this modified phage by the purified rabbit and guinea pig antibodies against (Pro-Gly-Pro)$_n$. This inactivation was completely prevented by preincubation of the purified antibodies with the ordered polymer.

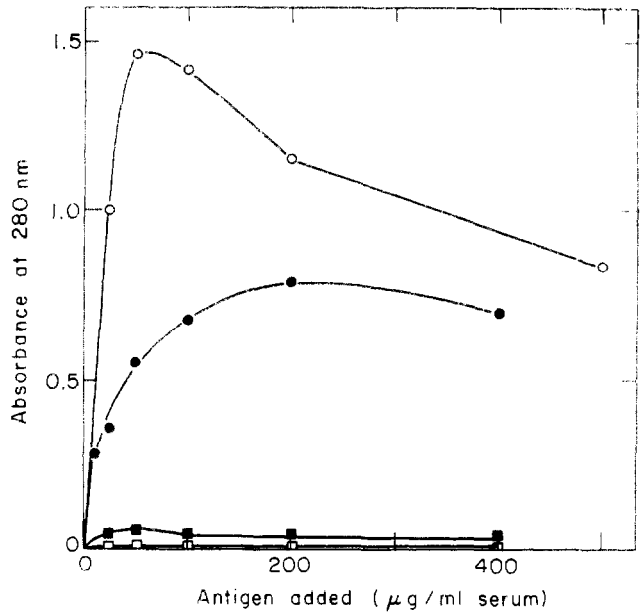

FIGURE 2: Precipitin curves of anti-(Pro-Gly-Pro)$_n$-ovalbumin serum with (Pro-Gly-Pro)$_n$-ovalbumin (○), (Pro-Gly-Pro)$_n$–RNase (●), ovalbumin (■), and Pro-Gly-Pro–RNase (□).

Purified goat anti-(Pro-Gly-Pro)$_n$–antibodies were fractionated on a Sephadex G-200 column. Antibody activity was detected both in the fraction sedimenting with 19 S and in the fraction sedimenting with 7 S, using the modified bacteriophage inactivation technique for assaying antibody activity.

Immune Response to (Pro-Gly-Pro)$_n$–Protein Conjugates. To increase the antibody production against the collagen-like ordered polymer, we conjugated the polymer to a carrier protein, and used such conjugates for immunization. The preparation of (Pro-Gly-Pro)$_n$–RNase and (Pro-Gly-Pro)$_n$–ovalbumin is described in the Materials and Methods section. The two conjugates contained respectively 10 and 16 mol of polymer/protein. Rabbits were immunized with (Pro-Gly-Pro)$_n$–ovalbumin. The antisera obtained gave relatively strong precipitin reactions both with the homologous immunogen and with the conjugate of the polymer with an unrelated protein, RNase (Figure 2). It is of interest to point out that this antiserum gave no precipitin reaction with the carrier protein, ovalbumin. The stronger reaction with the homologous (Pro-Gly-Pro)$_n$–ovalbumin, rather than with the heterologous (Pro-Gly-Pro)$_n$–RNase, reflects most probably the presence of antibodies against the polymer as well as of antibodies against antigenic determinants including both areas of the polymer and areas of ovalbumin. The ordered polymer inhibited this precipitation (Figure 3).

(Pro-Gly-Pro)$_n$–specific antibodies could be detected in the anti-(Pro-Gly-Pro)$_n$–ovalbumin sera also by immunological

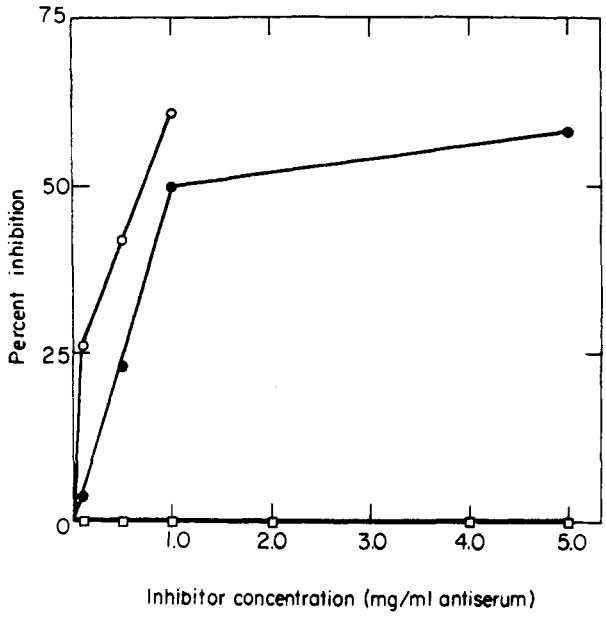

FIGURE 3: Inhibition of the precipitin reactions of anti-(Pro-Gly-Pro)$_n$-ovalbumin serum. Inhibition of precipitation with (Pro-Gly-Pro)$_n$-ovalbumin by (Pro-Gly-Pro)$_n$ of mol wt 1910 (○), and of precipitation with (Pro-Gly-Pro)$_n$-RNase by (Pro-Gly-Pro)$_n$ of mol wt 1910 (●), and by Pro-Gly-Pro (□).

techniques other than precipitin such as hemagglutination of (Pro-Gly-Pro)$_n$-coated sheep erythrocytes or (Pro-Gly-Pro)$_n$-RNase-coated erythrocytes, as well as by inactivation of (Pro-Gly-Pro)$_n$-modified bacteriophage preparations. The hemagglutination titer of anti-(Pro-Gly-Pro)$_n$-ovalbumin by sheep erythrocytes coated with (Pro-Gly-Pro)$_n$-RNase was 1/32,768 (Table I).

Both (Pro-Gly-Pro)$_n$-T4 and (Pro-Gly-Pro)$_n$-RNase-T4 were efficiently inactivated by antisera to the conjugate; inactivation of the latter is depicted in Figure 4. As can be seen in this figure, a 20,000-fold dilution of the antiserum gave about 90% inactivation of the phage. This inactivation was prevented by preincubation of the antisera with the ordered polymers (Figure 5).

Immune Response to Pro-Gly-Pro–Ovalbumin. The ordered periodic polymer (Pro-Gly-Pro)$_n$ possesses the rigid collagen-like structure. It was of interest to find out whether there is any resemblance between the polymer and the monomeric tripeptide in terms of immunological cross-reactions. For this purpose, Pro-Gly-Pro was attached both to ovalbumin (21 mol of tripeptide/protein) and to RNase (8 mol of tripeptide/protein). Pro-Gly-Pro–ovalbumin was totally in-

TABLE I: Specificity of Rabbit Antisera against (Pro-Gly-Pro)$_n$-ovalbumin and Pro-Gly-Pro–ovalbumin.[a]

	Erythrocytes Coated with		
Antiserum against	(Pro-Gly-Pro)$_n$-RNase	(Pro^{66},-Gly^{34})$_n$-RNase	Pro-Gly-Pro-RNase
(Pro-Gly-Pro)$_n$-ovalbumin	1:32768[b]	1:4096	1:4
Pro-Gly-Pro–ovalbumin	1:64	1:16	1:512

[a] The antisera were tested by the passive hemagglutination test with antigen-coated erythrocytes. [b] These numbers represent the hemagglutination titers.

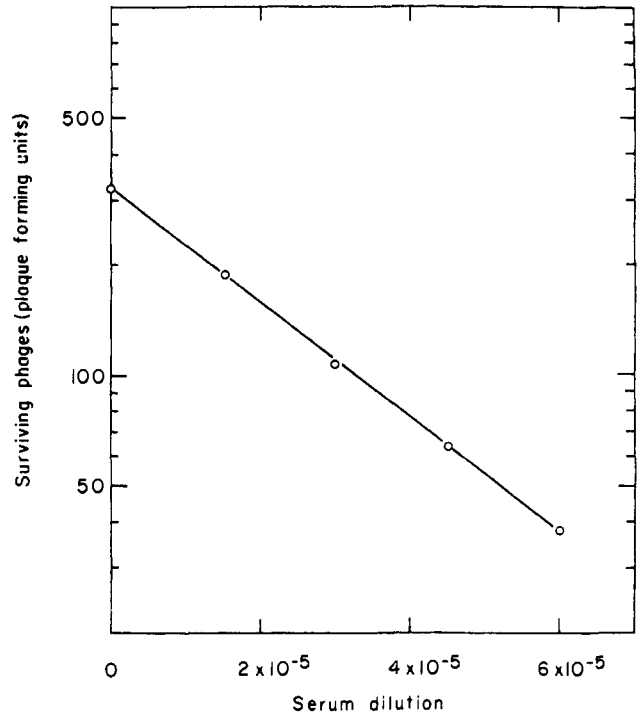

FIGURE 4: Inactivation of (Pro-Gly-Pro)$_n$-RNase-bacteriophage T4 conjugate by rabbit anti-(Pro-Gly-Pro)$_n$-ovalbumin serum.

capable of precipitating antibodies against the polymer, as seen in Figure 2 which shows the lack of cross-reaction between Pro-Gly-Pro–ovalbumin and antibodies against (Pro-Gly-Pro)$_n$-ovalbumin.

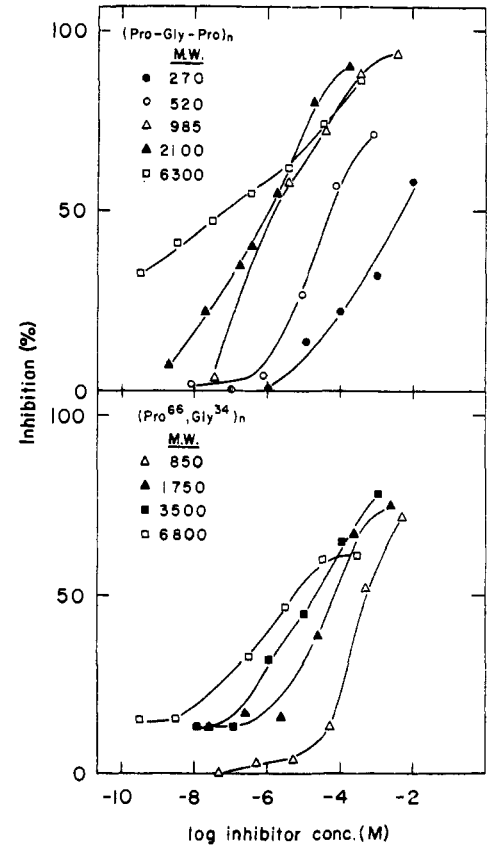

FIGURE 5: Inhibition of the inactivation of (Pro-Gly-Pro)$_n$-RNase-bacteriophage T4 by rabbit anti-(Pro-Gly-Pro)$_n$-ovalbumin serum with ordered (top) and random (bottom) polymers.

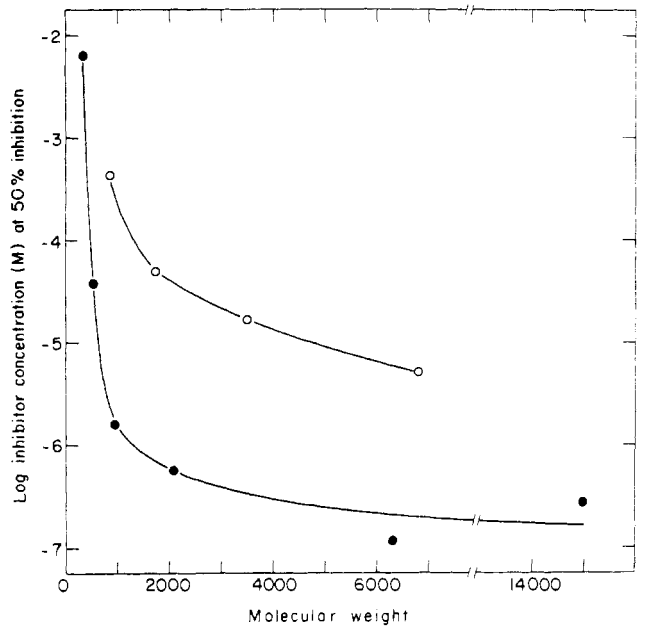

FIGURE 6: Molecular weight dependence of the inhibitory capacity of the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ (●) and the random polymer $(Pro^{66},Gly^{34})_n$ (○). The concentrations of inhibitor required for 50% inhibition of the inactivation of $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–T4 by anti-$(Pro\text{-}Gly\text{-}Pro)_n$ serum were derived from the data presented in Figure 5.

Antibodies to the conjugate of the tripeptide with ovalbumin were elicited in rabbits. The antibodies obtained could be precipitated by Pro-Gly-Pro–RNase. The antiserum contained 1.1 mg of precipitable anti-Pro-Gly-Pro–antibodies/1 ml of serum. This antiserum did not give any significant precipitation with the carrier protein, ovalbumin. The precipitin reaction of anti-Pro-Gly-Pro–ovalbumin with Pro-Gly-Pro–RNase was inhibited by the tripeptide. The ordered poylmers, $(Pro\text{-}Gly\text{-}Pro)_n$, also inhibited this precipitin reaction, but the efficiency of inhibited decreased with the increase in the molecular weight of the polymer.

Anti-Pro-Gly-Pro–ovalbumin sera had a titer of 1/512 when tested by hemagglutination of sheep erythrocytes coated with Pro-Gly-Pro–RNase (Table I). By this technique a cross-reaction with the ordered polymer was also detected.

Antigenic Specificity of Antibodies to the Collagen-like Polymer $(Pro\text{-}Gly\text{-}Pro)_n$. All the experiments, described earlier with antisera against the conjugate of the polymer with a carrier protein indicate that these antisera contain antibodies to the polymer which was conjugated, and that the specificity of these antibodies is like that of the antibodies obtained by immunization with the free polymer. Since the antibody titers are much higher in the anti-conjugate sera, many experiments for studying the antigenic specificity of the antibodies to the ordered polymer were performed with anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin serum.

The specificity of anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin antiserum toward ordered and random polymers of proline and glycine, of different molecular weights, was tested by the capacity of these polymers to inhibit the immunospecific inactivation of $(Pro\text{-}Gly\text{-}Pro)_n$-modified bacteriophage by the antiserum.

Role of the Polymer Size. In all cases (Figure 5) polymers of higher molecular weights are more efficient inhibitors. Moreover, within a wide range of molecular weights of the polymers tested, the ordered polymers are always much better inhibitors than the random ones. When the inhibitor concentrations required for 50% inhibition are plotted as a function of the molecular weight of the inhibitors (Figure 6), it can be seen that the ordered polymers are by about two orders of magnitude better than the random polymers of similar molecular weights. This suggests again that the affinity of the antibodies to conformation-dependent determinants is by far higher than to sequential stretches.

The curve describing the dependence of the inhibition on the molecular weight of the ordered polymer (Figure 6) seems to be composed of two regions. For small polymers there is a strong dependence on the molecular weight, but, as their size increases, the inhibitory capacity is not significantly augmented with the molecular weight.

Cross-Reactivity with the Tripeptide. The tripeptide Pro-Gly-Pro is a very poor inhibitor of the inactivation of $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–T4 by anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin serum, whereas the dipeptide Gly-Pro did not cause any inhibition at a concentration as high as 2.4 M (4 mg/ml). The low affinity of the antibodies to the ordered polymer toward the tripeptide was also confirmed by other techniques. The tripeptide Pro-Gly-Pro did not inhibit the precipitation of anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin by $(Pro\text{-}Gly\text{-}Pro)_n$–RNase (Figure 3), and also erythrocytes coated with Pro-Gly-Pro–RNase were not significantly agglutinated by anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin (Table I). On the other hand, antibodies to the tripeptide Pro-Gly-Pro *did* cross-react, when the reaction was followed by the hemagglutination technique, with the ordered polymer (Table I). It is of interest to point out that, whereas the tripeptide did not cross-react by passive hemagglutination with antibodies to the ordered polymer, it nevertheless cross-reacted with antibodies to the random polymer $(Pro^{66},Gly^{34})_n$.

Effect of Collagenase Digestion. Additional evidence for the conformation-dependent specificity of the antibodies to the ordered polymer came from studies with collagenase. It was first shown that both the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ and the random analog, $(Pro^{66},Gly^{34})_n$, are digested by collagenase. The digestion of the ordered polymer was recently described by Harper *et al.* (1972). When the polymers were digested with collagenase, and their inhibition of the inactivation of the modified phage was measured as a function of time of enzymatic digestion (Figure 7), it was found that the inhibitory capacity of the ordered polymer decreased markedly as a result of collagenase digestion, whereas the inhibitory capacity of the random polymer did not change significantly upon a similar digestion. After 4 hr of digestion the inhibitory capacity of both the ordered and random polymers become similar and is approximately of the same extent as that of the tripeptide (Figure 6). It seems that the collagenase effect on the ordered polymer is by virtue of losing its three-dimensional, collagen-like structure.

Cross-Reactions with Polyhexapeptides. So far all the polymers or peptides which were used to test the specificity of the anti-$(Pro\text{-}Gly\text{-}Pro)_n$ antibodies were of identical amino acid composition, namely L-proline and glycine in a residue molar ratio of 2:1, though different in molecular weights and sequence. The studies of the antigenic specificity were extended also to poly-L-proline and to several polymers of ordered hexapeptides which were shown to have a collagen-like conformation (Segal, 1969). Rabbit and mouse anti-polyproline antibodies, obtained by immunization with poly-L-proline (mol wt 12,100) (Gurari *et al.*, 1973) and kindly provided by Dr. D. Gurari, did not inactivate the $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–T4 bacteriophage. However, the inactivation of this modified bacteriophage by anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin could be

TABLE II: Inhibition of the Inactivation of $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–Bacteriophage T4 with Anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin by Means of Collagen-like Polyhexapeptides.

Inhibitor	Mol Wt	Inhibitor Concn (M) Needed for 50% Inhibn
$(Gly\text{-}Pro\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Pro)_n$ [a]	2100	5.6×10^{-7}
$(Gly\text{-}Pro\text{-}Ala\text{-}Gly\text{-}Pro\text{-}Pro)_n$	2600	1.3×10^{-5}
$(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Pro)_n$	2800	7.9×10^{-5}
$(Gly\text{-}Ala\text{-}Ala\text{-}Gly\text{-}Pro\text{-}Pro)_n$	2300	5.5×10^{-5}
$(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Ala)_n$	2400	1.7×10^{-3} [b]

[a] This is the ordered polytripeptide $(Pro\text{-}Gly\text{-}Pro)_n$. The following polyhexapeptides were obtained by polymerizing respectively the hexapeptides Pro-Ala-Gly-Pro-Pro-Gly, Ala-Pro-Gly-Pro-Pro-Gly, Ala-Ala-Gly-Pro-Pro-Gly, and Ala-Pro-Gly-Pro-Ala-Gly. [b] This concentration of inhibitor gave 48% inhibition.

weakly inhibited by a poly-L-proline of a low molecular weight (mol wt 4000). Poly-L-hydroxyproline did not give any inhibition. In addition, it should be noted that anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin did inactivate poly-L-prolyl bacteriophage T4 (Gurari *et al.*, 1973).

A series of four polyhexapeptides, $(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Pro)_n$, $(Gly\text{-}Pro\text{-}Ala\text{-}Gly\text{-}Pro\text{-}Pro)_n$, $(Gly\text{-}Ala\text{-}Ala\text{-}Gly\text{-}Pro\text{-}Pro)_n$, and $(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Ala)_n$, similar to $(Pro\text{-}Gly\text{-}Pro)_n$, but distinct in one or two of the proline residues within the repeating hexapeptide Gly-Pro-Pro-Gly-Pro-Pro being replaced by L-alanine, was synthesized by Segal (1969) in order to simulate actual collagen-type sequences and to study the relationship between sequence and physicochemical properties in the collagen fold. These polyhexapeptides were shown to be collagen like both in their properties in solution (Segal, 1959) as well as from X-ray diffraction measurements (Segal *et al.*, 1969). It was thus of interest to also test whether these polyhexapeptides have an antigenic specificity similar to that of the collagen-like polymer $(Pro\text{-}Gly\text{-}Pro)_n$. This was achieved by testing the inhibition caused by the polyhexapeptides of the reaction between $(Pro\text{-}Gly\text{-}Pro)_n$ and anti-$(Pro\text{-}Gly\text{-}Pro)_n$, using the modified phage immunoassay. As can be seen in Table II, the immunological cross-reactivity is in good agreement with the physicochemical studies. It seems that the inhibitory capacity of the polyhexapeptides is better when an imino acid (proline) follows each glycine (X in the Gly-X-Y triplet), as $(Gly\text{-}Pro\text{-}Ala\text{-}Gly\text{-}Pro\text{-}Pro)_n$ is a better inhibitor than $(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Pro)_n$ or $(Gly\text{-}Ala\text{-}Ala\text{-}Gly\text{-}Pro\text{-}Pro)_n$. Similarly, it was suggested by Segal (1969) that substitution of an alanyl for prolyl residue in the position following glycine (X) has a greater destabilizing effect than substitution in the position twice removed from glycine (Y). It is of interest that by the immunological assay for cross-reactivity with anti-$(Pro\text{-}Gly\text{-}Pro)_n$, the polyhexapeptide $(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Ala)_n$, is about two orders of magnitude a weaker inhibitor than the other three polyhexapeptides, although it was also found (Segal, 1969) to form stable collagen helices in solution. However, this polymer lacks any Gly-Pro-Pro sequences and was actually not expected to have a collagen-like structure (Segal, 1969).

Antigenic Specificity in Guinea Pigs. The immunological specificity of anti-$(Pro\text{-}Gly\text{-}Pro)_n$ was further assessed in guinea pigs by skin reactions. It was already shown by Borek *et al.* (1969) that there is significant cross-reactivity by skin reactions between the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ and the random one, $(Pro^{66},Gly^{34})_n$. These studies were now confirmed and extended. Guinea pigs were immunized respectively with the tripeptide Pro-Gly-Pro, with the ordered polymers, $(Pro\text{-}Gly\text{-}Pro)_n$, of different molecular weights, with a conjugate of the tripeptide with a protein carrier, Pro-Gly-Pro-ovalbumin, and with a conjugate of the ordered polymer with a protein $(Pro\text{-}Gly\text{-}Pro)_n$–RNase. The animals were skin tested with the tripeptide Pro-Gly-Pro and with the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ (Table III). The higher the molecular weight of the immunizing polymer, the stronger the intensity of the immediate skin reactions elicited with $(Pro\text{-}Gly\text{-}Pro)_n$. The free tripeptide, as expected, did not induce any immunological response. However, when guinea pigs were immunized with Pro-Gly-Pro–ovalbumin, skin cross-reactions with the ordered polymer were detected (Table III). Practically all the guinea pigs immunized with $(Pro\text{-}Gly\text{-}Pro)_n$ gave delayed reactions with the polymer. In some animals immunized with the ordered polymer, the tripeptide Pro-Gly-Pro elicited a very weak delayed reaction. These weak reactions were detected mainly in guinea pigs immunized with polymers of low molecular weight.

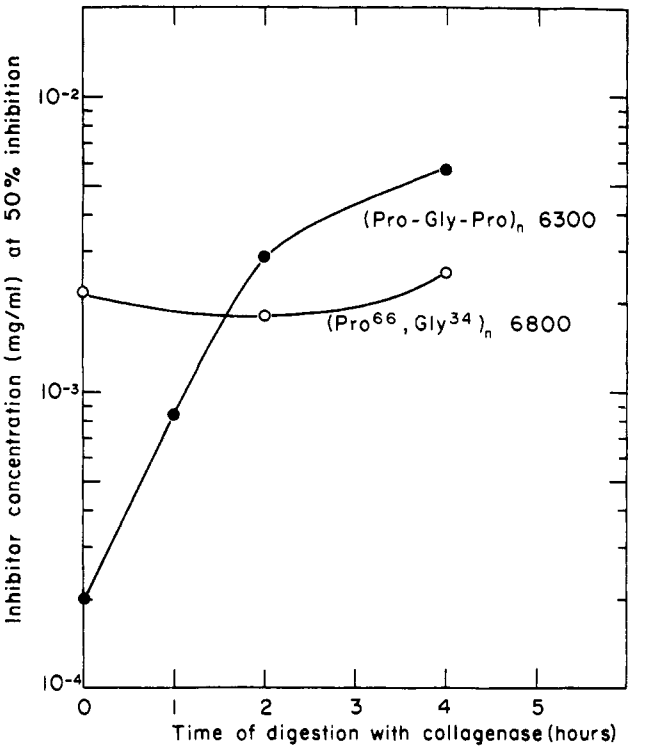

FIGURE 7: The effect of collagenase digestion of the ordered $(Pro\text{-}Gly\text{-}Pro)_n$ and the random $(Pro^{66},Gly^{34})_n$ on their inhibitory capacity of the inactivation of $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–T4 by rabbit anti-$(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin serum. Samples of $(Pro\text{-}Gly\text{-}Pro)_n$ (mol wt 6300) and of $(Pro^{66},Gly^{34})_n$ (mol wt 6800) were incubated at 37° with collagenase (2% by weight of the polymer). Aliquots were removed from the digestion mixture at various times of incubation and diluted into Ca^{2+}-free medium for inhibition.

Discussion

Steric conformation of the immunogenic macromolecule plays a crucial role in determining the specificity of the antibodies produced. This has been apparent since the early studies showing very little cross-reaction, if at all, between the same proteins in native and in denatured state. More re-

TABLE III: Immune Response in Guinea Pigs to $(Pro\text{-}Gly\text{-}Pro)_n$ and to Pro-Gly-Pro as Determined by Skin Reactions.

Immunizing Antigen	Mol Wt	Test Antigen	Skin Reactions: Day 9 Immediate (2 hr)	Day 9 Delayed (24 hr)	Day 18 Immediate (2hr)	Day 18 Delayed (24 hr)	Day 32 Immediate (2 hr)	Day 32 Delayed (24 hr)
$(Pro\text{-}Gly\text{-}Pro)_n$	915	Pro-Gly-Pro	0/5[a]	2/5 (5.5)[b]	0/5	2/5 (6)	0/5	2/5 (6)
		$(Pro\text{-}Gly\text{-}Pro)_n$[c]	0/5	4/5 (9)	2/5 (11)	3/5 (9)	1/5 (11)	3/5 (15)
$(Pro\text{-}Gly\text{-}Pro)_n$	1910	Pro-Gly-Pro	0/5	2/5 (8)	3/5 (8)	3/5 (9)	4/5 (6.5)	2/5 (6)
		$(Pro\text{-}Gly\text{-}Pro)_n$	2/5 (10)	5/5 (13.5)	4/5 (10)	5/5 (10.5)	4/5 (19)	4/5 (17)
$(Pro\text{-}Gly\text{-}Pro)_n$	6300	Pro-Gly-Pro	0/5	0/5	0/5	0/5	0/5	0/5
		$(Pro\text{-}Gly\text{-}Pro)_n$	4/5 (11)	5/5 (11.5)	5/5 (20)	5/5 (20)	5/5 (22.5)	5/5 (14)
$(Pro\text{-}Gly\text{-}Pro)_n$–RNase		Pro-Gly-Pro	1/5 (7)	0/5	0/5	1/5 (10)	3/5 (6)	1/5 (6)
		$(Pro\text{-}Gly\text{-}Pro)_n$	5/5 (12)	4/5 (9)	4/5 (16)	5/5 (16)	5/5 (17.5)	4/5 (15)
Pro-Gly-Pro		Pro-Gly-Pro	0/5	0/5	0/5	0/5	0/5	0/5
		$(Pro\text{-}Gly\text{-}Pro)_n$	0/5	0/5	0/5	0/5	0/5	0/5
Pro-Gly-Pro–ovalbumin		Pro-Gly-Pro	0/5	4/5 (8)	2/5 (6)	4/5 (7.5)	3/5 (9)	3/5 (8)
		$(Pro\text{-}Gly\text{-}Pro)_n$	3/5 (8.6)	4/5 (11)	3/5 (11)	5/5 (9)	4/5 (15)	5/5 (14)

[a] Ratio of responders to total number of animals. [b] Average reaction diameter in mm. Skin reactions with an average diameter of 5 mm or less were considered negative. [c] Mol wt 6300.

cently, it was shown that the same tripeptide, Tyr-Ala-Glu, may be incorporated into a synthetic antigen, leading to the production of antibodies against either "sequential" or "conformational" determinants. Thus, when the tripeptide is attached to a branched polymer, the antibodies obtained are directed against a sequential determinant, whereas when the tripeptide is present as building block of a periodic polymer which is α helical, the antibodies are directed against conformational determinants (Schechter *et al.*, 1971a,b).

In the present paper we have analyzed in detail the immunogenicity and antigenic specificity of another periodic polymer, of the tripeptide Pro-Gly-Pro. Previous work from our laboratory has indicated that this polymer, similar to collagen in its physicochemical properties in the solid state and in solution (Traub and Yonath, 1965, 1966; Engel *et al.*, 1966), resembles collagen also when tested by immunological criteria (Borek *et al.*, 1969). We have now investigated the immunochemical properties of the ordered polymer, $(Pro\text{-}Gly\text{-}Pro)_n$, and compared them with the random polymer of a similar composition, $(Pro^{66},Gly^{34})_n$, as well as with an antigen in which the tripeptide Pro-Gly-Pro is attached to a protein carrier.

The ordered sequence polytripeptide $(Pro\text{-}Gly\text{-}Pro)_n$ shows the capacity to provoke, in guinea pigs, rabbits, and goats, antibodies which could be detected and characterized by various techniques. The use of the conjugate $(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin for immunization increased markedly the formation of antibodies with specificity directed to $(Pro\text{-}Gly\text{-}Pro)_n$ (Figures 2–4; Table I). For a sensitive detection and characterization of anti-$(Pro\text{-}Gly\text{-}Pro)_n$-antibodies we made use of the chemically modified bacteriophage technique (Mäkelä, 1966; Haimovich and Sela, 1966). $(Pro\text{-}Gly\text{-}Pro)_n$ was attached to bacteriophage T4 either directly, by making use of difluorodinitrobenzene as the cross-linking agent, or *via* RNase as a carrier. The $(Pro\text{-}Gly\text{-}Pro)_n$-coated phage preparations thus obtained could be efficiently inactivated by anti-$(Pro\text{-}Gly\text{-}Pro)_n$-antibodies (Figures 1 and 4), and this inactivation may be inhibited by cross-reactive haptens and antigens (Figures 5 and 6).

The collagen-like conformation of $(Pro\text{-}Gly\text{-}Pro)_n$-oligopeptides depends on the size of the polymer (Sakakibara *et al.*, 1968; Kobayashi *et al.*, 1970). It was of interest to find out whether this is reflected also by immunological assays. Indeed, the specificity of the antibodies to $(Pro\text{-}Gly\text{-}Pro)_n$ was studied by a series of inhibition experiments in which the inactivation of $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–T4 preparation with anti$(Pro\text{-}Gly\text{-}Pro)_n$-antibodies was inhibited by means of $(Pro\text{-}Gly\text{-}Pro)_n$ of increasing molecular weights (Figures 5 and 6). The low molecular weight polymers are weaker inhibitors than the ones with relatively high molecular weight. Larger amounts of the low molecular weight inhibitors are required in order to obtain the extent of inhibition caused by the high molecular weight polymers. The molecular weight dependence of the efficiency of inhibition is more profound up to a molecular weight of 2000, after which there is no marked increase in the efficiency of inhibition with a further increase in the molecular weight. However, it should be noted here that the antibodies tested were elicited by a conjugate in which $(Pro\text{-}Gly\text{-}Pro)_n$ of a molecular weight of 2000 was attached to the carrier. It is possible that with antibodies against higher molecular weight stretches of $(Pro\text{-}Gly\text{-}Pro)_n$ a larger increase in the efficiency of inhibition with molecular weight could be obtained.

The free tripeptide, Pro-Gly-Pro itself, is a very poor inhibitor (Figure 5) and was essentially nonreactive with anti-$(Pro\text{-}Gly\text{-}Pro)_n$-antibodies by precipitation reactions or passive hemagglutination (Figures 2 and 3; Table I). From specificity studies in guinea pigs (Table III) it was shown that there is a weak cross-reaction with the tripeptides in guinea pigs immunized with low molecular weight $(Pro\text{-}Gly\text{-}Pro)_n$. However, such cross-reaction was completely abolished when the immunogen was of a higher molecular weight (mol wt 6300).

In an early study (Engel *et al.*, 1966) evidence was brought that $(Pro\text{-}Gly\text{-}Pro)_n$ is in a stable helical conformation in solution. This is due mainly to the restriction of the rotational freedom of the bonds in the prolyl–proline moiety within the tripeptide Gly-Pro-Pro. The helicity increases with increasing molecular weight of the polymer. The consideration of this aspect may be very helpful in understanding the nature of the lack of cross-reactivity observed between the periodic polytripeptide and its building block Pro-Gly-Pro. Immunization with the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$

would lead to production of antibodies with restricted specificity to conformation-dependent determinants and, therefore, these antibodies would not recognize the tripeptide sequence. On the other hand, the immunization with the tripeptide Pro-Gly-Pro conjugated to ovalbumin elicited formation of antibodies which weakly cross-reacted with the ordered polytripeptide $(Pro\text{-}Gly\text{-}Pro)_n$ (Table I). This is probably due to some sequences at the edges of the polymer, or even in the chain, which are not in the collagen-like conformation. It seems that, at least in the high molecular weight polymers, such stretches are not immunopotent.

The studies reported here show that the specificity of the antibodies to the ordered collagen-like polymer is directed to the unique conformation of the polymer rather than to its specific sequences. This conclusion is based on studies of the antigenic specificity of the antibodies toward ordered and random polymers of proline and glycine in a molar residue ratio of 2:1 and toward a series of polyhexapeptides containing proline, glycine, and alanine, as well as from the effect of a collagenase digestion on the inhibitory capacity of the polymers. The ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ is by at least two orders of magnitude more efficient than the random $(Pro^{66},Gly^{34})_n$ of similar amino acid composition, in reacting with antibodies to the ordered polymer (Figures 5 and 6). Moreover, the efficiency of the reaction of these antibodies with a series of collagen-like polyhexapeptides (Table II) corroborates the collagen similarity of these polyhexapeptides. The experiments with collagenase show that, while collagenase digestion does not affect the reaction of the antibodies with the random copolymer which does not have the collagen-like conformation, it nevertheless affects markedly the reaction with the ordered polymer (Figure 7), probably by destroying its original conformation. As has been reported earlier (Borek *et al.*, 1969), and as will be discussed in the following paper (Maoz *et al.*, 1973), the $(Pro\text{-}Gly\text{-}Pro)_n$ system cross-reacts also with natural collagens. Such cross-reaction is undoubtedly due to the higher order structure of this polymer.

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