On Immunological Cross-Reactions between the Synthetic Ordered Polypeptide (L-Pro-Gly-L-Pro)$_n$ and Several Collagens†

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ABSTRACT: The synthetic ordered polypeptide (L-Pro-Gly-L-Pro)$_n$ elicits in guinea pigs an immune response which cross-reacts with collagens of several species by delayed and immediate skin reactions. The broad range cross-reactivity with natural collagens includes *inter alia* cross-reaction with collagen of the same animal species, namely guinea pig skin collagen. No immunological cross-reactivity was detected between the random copolymer (L-Pro^{66},Gly^{34})$_n$ and collagens. The cellular cross-reactions with collagen, manifested by delayed type sensitivity, precede in all cases the immediate type. The cross-reactivity with collagens increases with the molecular weight of the immunizing polymer.

The ordered polytripeptide (Pro-Gly-Pro)$_n$ and collagen from the cuticle of the invertebrate *Ascaris lumbricoides* show strong cross-reactions in rabbits and guinea pigs. The specificity of the humoral cross-reactions in rabbits was studied by several immunological methods. The cross-reactivity of anti-*Ascaris* collagen with the ordered periodic polymer (Pro-Gly-Pro)$_n$ is much stronger than with the random copolymer (Pro^{66},Gly^{34})$_n$, and depends on the molecular weight of the polymers. It was shown that antisera to *Ascaris* collagen contain two types of antibodies: specific to common collagen structure and species specific. The antibodies with common collagen specificity were immunospecifically bound to an immunoadsorbent of the collagen-like polytripeptide.

The periodic polypeptide (L-Pro-Gly-L-Pro)$_n$ was shown to have a collagen-like conformation in its three-dimensional structure both in solution (Engel *et al.*, 1966) and in its X-ray diffraction pattern (Traub and Yonath, 1965, 1966). The immunological properties of this polymer were studied earlier (Borek *et al.*, 1969) and were extended recently to a more detailed characterization of the immune response to this polymer (Maoz *et al.*, 1973a). Borek *et al.* (1969) were able to demonstrate a weak cross-reactivity between antiserum to (Pro-Gly-Pro)$_n$ and collagens of several animal species. Moreover, a slight cross-reactivity was also observed by passive cutaneous anaphylaxis (pca) reaction between guinea pig skin collagen and guinea pig antibodies to (Pro-Gly-Pro)$_n$ which implies the induction of antibodies, cross-reacting with an autologous collagen, by means of a synthetic antigen.

Immunological cross-reactions between collagens and synthetic polypeptides containing glycine and proline were also studied by other investigators (Jasin and Glynn, 1965a,b; Kettman *et al.*, 1967).

We report here on studies of immunological cross-reactivity between collagens and synthetic collagen-like polypeptides, both in the humoral and the cellular levels of the immune response. A previous report (Borek *et al.*, 1969) demonstrated only a weak cross-reaction between native collagen and the synthetic model. Since then we were able to increase the intensity of the immune response to (Pro-Gly-Pro)$_n$ and also to apply the sensitive technique of inactivation of chemically modified bacteriophage in order to detect antibody production (Maoz *et al.*, 1973a). This enabled us to extend the studies of possible cross-reactivity with mammalian collagens. The specificity of anti-(Pro-Gly-Pro)$_n$ was further tested with collagens from the cuticle of the parasite *Ascaris lumbricoides* and also from earthworm cuticle, in order to find out whether the cross-reactivity of the antibodies to the ordered polymer extends also to invertebrate collagens.

Materials and Methods

Antigens. The ordered collagen-like synthetic polytripeptide (L-Pro-Gly-L-Pro)$_n$ and the random copolymer (L-Pro^{66},-Gly^{34})$_n$ were prepared and characterized as described in the preceding paper (Maoz *et al.*, 1973a). Acid-soluble collagen from rat tail tendon was prepared according to Piez *et al.* (1963). Guinea pig skin collagen was a gift from Dr. D. Michaeli. Reduced and carboxymethylated (RCM) *Ascaris* cuticle collagen (McBride and Harrington, 1967) was a gift from Dr. W. F. Harrington. The specific rotation of this sample immediately after dissolving in 0.15 M NaCl–0.05 M sodium acetate (pH 4.8) was $[\alpha]^{15}_{210\,nm}$ 21,000. Earthworm cuticle collagen (Josse and Harrington, 1964) was a gift from Dr. E. Adams.

Antisera. Immunization of rabbits and guinea pigs with the ordered polymer, (Pro-Gly-Pro)$_n$, the random copolymer, (Pro^{66},Gly^{34})$_n$, and with conjugates of the ordered polymer with carrier proteins was described in the preceding paper (Maoz *et al.*, 1973a). Rabbit antisera against *Ascaris* cuticle collagen, and the reduced carboxymethylated (RCM) form of it, were prepared earlier (Fuchs and Harrington, 1970; Maoz *et al.*, 1971). Antisera against rat tail tendon collagen was prepared in rabbits by multiple intradermal injections of the antigen in complete Freund's adjuvant. Each rabbit received two injections, 12 days apart, each containing 2 mg of antigen. Rabbit antisera to guinea pig skin collagen and to guinea pig gelatin were gifts from Dr. D. Michaeli.

Immunological Methods. Quantitative precipitin tests were prepared as described earlier (Fuchs and Sela, 1963).

Antisera were examined by the passive cutaneous anaphylaxis (pca) reaction (Ovary, 1958) with the modifications described by Ben-Efraim *et al.* (1964) using 100 μg of the test

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antigen per animal. Sera of nonimmunized rabbits and guinea pigs served as controls.

Passive hemagglutination tests were performed with formalinized and tanned sheep erythrocytes coated with the respective antigen (Herbert, 1967) on disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) (Kabat, 1968).

The preparation of (Pro-Gly-Pro)$_n$–RNase–bacteriophage T4 conjugate was described in the preceding paper (Maoz *et al.*, 1972a). Inactivation of the modified bacteriophage preparation and the inhibition of such inactivation with different inhibitors were performed as described elsewhere (Haimovich *et al.*, 1970a,b).

Skin tests in immunized guinea pigs (immediate or delayed) were performed according to Borek *et al.* (1969).

Results

It was previously reported (Borek *et al.*, 1969) that there is a weak cross-reactivity by pca reactions between anti-(Pro-Gly-Pro)$_n$ and ichthyocol (fish collagen), rat skin collagen, and guinea pig skin collagen. The ability of a synthetic antigen to elicit an immune response which cross-reacts with natural collagens is of special interest and may be helpful in studying structure and function relationships in collagens. For this purpose, the immunological cross-reactivity between synthetic collagen-like polypeptides and collagens of different species was further assessed.

Cellular and Humoral Cross-Reactions. Guinea pigs were immunized with the ordered polypeptide (Pro-Gly-Pro)$_n$ and the random copolymer (Pro^{66},Gly^{34})$_n$, and were skin tested 10 days later. Skin tests were performed with both polymers as well as with natural collagens. As can be seen in Table I, guinea pigs which were immunized with the ordered polymer gave delayed type skin reactions with several natural collagens, such as *Ascaris* cuticle collagen, guinea pig skin collagen, and rat tail tendon collagen, whereas animals immunized with the random copolymer did not give such cross-reactions. No humoral cross-reaction between the natural collagens and the synthetic polypeptides was detected by immediate type skin reactions 10 days after the immunization. It seems that the cellular type of cross-reaction, manifested by the delayed reaction, precedes the appearance of cross-reacting antibodies. As shown in Table I, the immediate reactions were weaker than the delayed ones even with the homologous antigen. In other experiments, when the animals were skin tested at later times after immunization, immediate cross-reactions with collagens were recorded, besides the delayed cross-reactions (see Table II).

Role of the Size of Immunogen. It was noted in the preceding paper that the collagen-like conformation of (Pro-Gly-Pro)$_n$ depends on the size of the polymer. The ability of collagens to give skin reactions in animals immunized with such polymers was tested as a function of the size of the immunogen. Guinea pigs were immunized with (Pro-Gly-Pro)$_n$ of different molecular weights, and with the tripeptide Pro-Gly-Pro. The animals were skin tested with (Pro-Gly-Pro)$_n$ (mol wt 6300), with the tripeptide Pro-Gly-Pro, and with three natural collagens. The results obtained show that the cross-reactivity with collagens depends clearly on the molecular weight of the immunizing polypeptide (Table II). The tripeptide, as expected, did not induce any detectable immune response with the homologous or any of the other antigens tested. No cross-reactions with collagens in animals immunized with (Pro-Gly-Pro)$_n$ of a low molecular weight (mol wt 915) could be detected. When preparations of higher molecular weight of (Pro-Gly-Pro)$_n$ were used for immunization, a strong cross-reactivity of both immediate and delayed types was observed with collagens. On the other hand, guinea pigs immunized with the high molecular weight (Pro-Gly-Pro)$_n$ failed to cross-react with the tripeptides, whereas a weak reaction with Pro-Gly-Pro was recorded in animals immunized with the low molecular weight (Pro-Gly-Pro)$_n$ (Table II). These results corroborated the finding that the collagen-like conformation of (Pro-Gly-Pro)$_n$ depends on the molecular weight of the polymer (Maoz *et al.*, 1973a).

TABLE I: Cross-Reactions between Natural Collagens and Collagen-like Synthetic Polypeptide as Determined by Skin Reactions in Guinea Pigs 10 Days after Immunization.

	Immunogen			
	(Pro-Gly-Pro)$_n$[a]		(Pro^{66},Gly^{34})$_n$	
	Skin Reactions[b]			
Test Antigen	Immediate (2 hr)	Delayed (24 hr)	Immediate (2 hr)	Delayed (24 hr)
(Pro-Gly-Pro)$_n$	3/8 (13)	8/8 (12)	2/3 (12)	3/3 (12)
(Pro^{66},Gly^{34})$_n$	0/3	8/8 (6)	2/3 (12)	3/3 (12)
Guinea pig skin collagen	0/3	8/8 (7)	0/3	0/3
RCM *Ascaris* collagen[c]	0/3	8/8 (10)	0/3	0/3
Rat tail tendon collagen	0/3	8/8 (6)	0/3	0/3

[a] Molecular weight of (Pro-Gly-Pro)$_n$ and (Pro^{66},Gly^{34})$_n$ used in this experiment is 6300 and 6800, respectively. [b] Ratio of responders to total numbers of animals. Numbers in parentheses give the average reaction diameter in mm. Reactions with an average diameter of 5 mm or less were considered negative. [c] Reduced and carboxymethylated (RCM) *Ascaris* cuticle collagen.

Specificity of Humoral Cross-Reactions with Collagens. Since (Pro-Gly-Pro)$_n$ as such seemed to be a rather weak immunogen, and antibodies could be detected only after their immunospecific isolation (Maoz *et al.*, 1973a), or by sensitive skin reactions (Tables I and II; Borek *et al.*, 1969), it was not surprising that this system was not appropriate for looking for cross-reactivity with natural collagens on a humoral level. The availability of precipitating anti-(Pro-Gly-Pro)$_n$–antibodies elicited by immunization with (Pro-Gly-Pro)$_n$–protein conjugates allowed the study of the cross-reactivity of such antibodies with collagens either by precipitation reactions or by the sensitive modified phage immunoassay. Anti-(Pro-Gly-Pro)$_n$–ovalbumin serum (Maoz *et al.*, 1973a) was reacted with rat tail collagen, guinea pig skin collagen, earthworm cuticle collagen, and RCM *Ascaris* cuticle collagen. Of all these collagens tested only the collagen from *Ascaris* cross-precipitated with anti-(Poly-Gly-Pro)$_n$–antibodies (Figure 1). The precipitation was about 16% of the antibodies which could be precipitated by (Pro-Gly-Pro)$_n$–ovalbumin or 38% of the amount or antibodies which could be precipitated by the heterologous antigen (Pro-Gly-Pro)$_n$–RNase. In a more sensitive assay, inhibition of the inactivation of (Pro-Gly-Pro)$_n$–RNase–T4 preparations by anti-(Pro-Gly-Pro)$_n$–ovalbumin was tested with different collagens.

TABLE II: Correlation between the Size of the Immunogen and the Cross-Reactions of Natural Collagens with Synthetic Collagen-like Polytripeptides.

Test Antigen	No. of Days after Immunization	Immunogen: Pro-Gly-Pro — Skin Reactions[a] Immediate (2 hr)	Pro-Gly-Pro Delayed (24 hr)	(Pro-Gly-Pro)$_n$ Mol Wt 915 Immediate (2 hr)	(Pro-Gly-Pro)$_n$ Mol Wt 915 Delayed (24 hr)	(Pro-Gly-Pro)$_n$ Mol Wt 1910 Immediate (2 hr)	(Pro-Gly-Pro)$_n$ Mol Wt 1910 Delayed (24 hr)	(Pro-Gly-Pro)$_n$ Mol Wt 6300 Immediate (2 hr)	(Pro-Gly-Pro)$_n$ Mol Wt 6300 Delayed (24 hr)
Pro-Gly-Pro	9	0/5	0/5	0/5[b]	2/5 (6)[c]	0/5	3/5 (8)	0/5	0/5
(Pro-Gly-Pro)$_n$		0/5	0/5	0/5	5/5 (9.5)	2/5 (10)	5/8 (13.5)	4/5 (11)	5/5 (14.5)
RCM *Ascaris* collagen[e]		0/5	0/5	0/5	0/5	0/5	3/5 (6)	0/5	5/5 (9.5)
Guinea pig skin collagen		0/5	0/5	0/5	0/5	0/5	1/5 (10)	0/5	3/5 (6)
Rat tail tendon collagen		0/5	0/5	0/5	0/5	0/5	4/5 (7)	0/5	4/5 (8)
Pro-Gly-Pro	18	0/5	0/5	0/5	2/5 (6)	3/5 (8)	3/5 (9)	0/5	0/5
(Pro-Gly-Pro)$_n$		0/5	0/5	2/5 (11)	3/5 (9)	4/5 (10)	5/5 (16.5)	5/5 (20)	5/5 (20)
RCM *Ascaris* collagen		0/5	0/5	0/5	0/5	2/5 (9.5)	5/5 (11.5)	5/5 (15)	5/5 (16)
Guinea pig skin collagen		0/5	0/5	0/5	0/5	0/5	2/5 (13)	1/5 (10)	4/5 (9)
Rat tail tendon collagen		0/5	0/5	0/5	0/5	0/5	3/5 (9)	2/5 (10)	3/5 (7)
Pro-Gly-Pro	32	0/5	0/5	0/5	2/5 (6)	4/5 (6.5)	2/5 (6)	0/5	0/5
(Pro-Gly-Pro)$_n$		0/5	0/5	1/5 (11)	3/5 (15)	4/5 (19)	4/5 (17)	5/5 (22.5)	5/5 (14)
RCM *Ascaris* collagen		0/5	0/5	0/5	0/5 (15)	4/5 (11)	4/5 (9)	5/5 (15.5)	4/5 (14.5)
Guinea pig skin collagen		0/5	0/5	0/5	0/5	2/5 (8)	3/5 (9)	5/5 (13.6)	3/5 (11)
Rat tail tendon collagen		0/5	0/5	0/5	0/5	2/5 (6)	3/5 (7)	5/5 (13)	3/5 (7)

[a] Control animals that were injected with buffered saline and complete Freund's adjuvant gave negative reaction with all the test antigens. [b] Ratio of responders to total number of animals. [c] Average reaction diameter in mm. Skin reactions (immediate or delayed) with an average diameter of 5 mm or less were considered negative. [d] Molecular weight 6300. [e] Reduced and carboxymethylated (RCM) *Ascaris* cuticle collagen.

Again, of all these collagens, at the concentrations tested, only the collagen from *Ascaris* showed significant inhibition (Figure 2). Cross-reactions between anti-(Pro-Gly-Pro)$_n$-antibodies and RCM *Ascaris* collagen was observed also by passive hemagglutination.

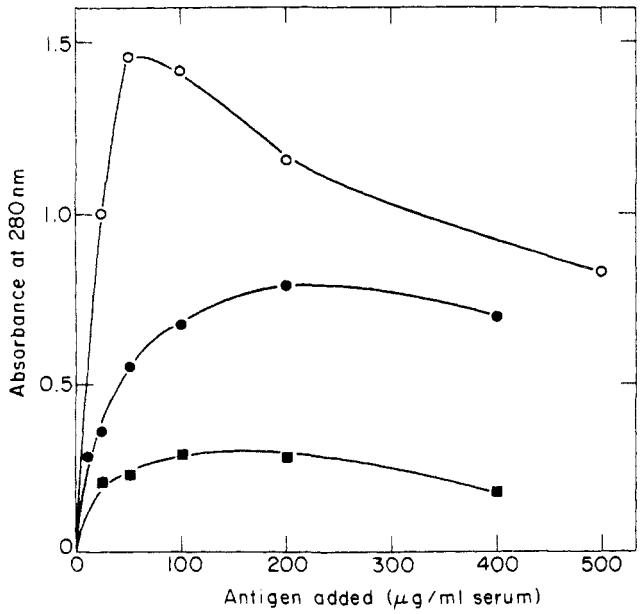

FIGURE 1: Precipitin curves of anti-(Pro-Gly-Pro)$_n$-ovalbumin serum with (Pro-Gly-Pro)$_n$-ovalbumin (○), (Pro-Gly-Pro)$_n$-RNase (●), and reduced and carboxymethylated (RCM) *Ascaris* collagen (■).

Cross-Reactivity of Anti-Ascaris Collagen Antibodies. In order to study the reverse cross-reactivity of antisera to collagens of different species with the synthetic collagen-like polypeptide, the ability of anti-collagen antisera to inactivate (Pro-Gly-Pro)$_n$-RNase-T4 bacteriophage preparation or to cause a positive pca reaction was tested. Rabbit antisera against rat tail tendon collagen, guinea pig skin collagen, gelatin (prepared from guinea pig skin collagen), earthworm collagen, and *Ascaris* cuticle collagen were reacted with the modified bacteriophage preparation. Of all sera tested, only anti-*Ascaris* collagen inactivated the (Pro-Gly-Pro)$_n$-RNase-T4 preparation (Figure 3). This antiserum also gave a positive pca reaction with (Pro-Gly-Pro)$_n$ (Table III). (Pro-Gly-Pro)$_n$-ovalbumin cross-reacts both with anti-*Ascaris* cuticle collagen and with anti-RCM *Ascaris* collagen (Figure 4) and precipitates 38 and 20%, respectively, of the precipitable antibodies in these two antisera.

It was reported by Steffen *et al.* (1968) that antibodies of diverse specificities may be raised by collagen (in their case it was calf collagen), namely, common collagen antibodies and species specific antibodies. It was of interest to find out whether the fraction of antibodies in anti-*Ascaris* collagen serum reacting with the ordered polymer (Pro-Gly-Pro)$_n$ reflects the common collagen specific antibodies. Anti-*Ascaris* collagen serum was adsorbed on (Pro-Gly-Pro)$_n$-Sepharose immunoadsorbent (Maoż *et al.*, 1973a) and the antisera before and after adsorption were tested for their capacity to give pca reaction with (Pro-Gly-Pro)$_n$ or the homologous antigen, *Ascaris* collagen. As can be seen in Table III, cross-reacting antibodies with (Pro-Gly-Pro)$_n$ in the antiserum to

TABLE III: Passive Cutaneous Anaphylaxis Reactions in Guinea Pigs with Anti-*Ascaris* Collagen Serum before and after Absorption on (Pro-Gly-Pro)$_n$–Sepharose.

Antiserum to	Serum Dilution	Antigen: (Pro-Gly-Pro)$_n$	Antigen: *Ascaris* Cuticle Collagen[a]
(Pro-Gly-Pro)$_n$-ovalbumin, nonabsorbed	1/1000	3/3[b] (16)[c]	3/3 (16.6)
(Pro-Gly-Pro)$_n$-ovalbumin, absorbed	1/10		
Ascaris cuticle collagen, nonabsorbed	1/2000	3/3 (12.6)	3/3 (13.3)
Ascaris cuticle collagen, absorbed	1/2000		
Ascaris cuticle collagen, absorbed	1/200		3/3 (16)

[a] Reduced and carboxymethylated. [b] Ratio of responders to total number of animals. [c] The average reaction diameter in mm. Reactions with an average diameter of 5 mm or less were considered as negative.

Ascaris collagen were adsorbed on the immunoadsorbent. However, this antiserum could still react strongly with the homologous antigen.

The specificity of the cross-reaction between collagen-like polypeptides and anti-*Ascaris* collagen serum was studied by modified phage technique. The inactivation of (Pro-Gly-Pro)$_n$–RNase–T4 bacteriophage by rabbit anti-*Ascaris* collagen serum was inhibited by the ordered polymer (Pro-Gly-Pro)$_n$ or by the random copolymer of proline and glycine (Pro^{66},Gly^{34})$_n$ of different molecular weights. As can be seen in Figure 5 the ordered collagen-like polymer is by at least two orders of magnitude a better inhibitor than the random copolymer, and the inhibitory capacity of the ordered polymer increases with its molecular weight. This inactivation reaction was also inhibited by a series of synthetic collagen-like polyhexapeptides containing proline, glycine, and alanine (Table IV). It was shown in the preceding paper (Maoz *et al.*, 1973a) that the immunological specificity of these polyhexapeptides corroborates their physicochemical features as measured in aqueous solutions (Segal, 1969) and in the solid state (Segal *et al.*, 1969). Similarly, as shown in Table IV, the immunological specificity with anti-*Ascaris* collagen follows the same pattern. Polyhexapeptides which were reported to be more collagen like exhibited a more efficient inhibition of the inactivation of (Pro-Gly-Pro)$_n$–RNase–T4 by anti-*Ascaris* collagen.

Discussion

The main finding described in the present paper is that a synthetic repeating ordered polytripeptide, (Pro-Gly-Pro)$_n$, provokes in experimental animals an immune response which is cross-reactive with collagens of several species both at the cellular and at the humoral level (Table I and II). An early report (Borek *et al.*, 1969) described cross-reactions of anti-(Pro-Gly-Pro)$_n$ with several collagens. The nature of this immunological cross-reactivity has now been investigated in detail.

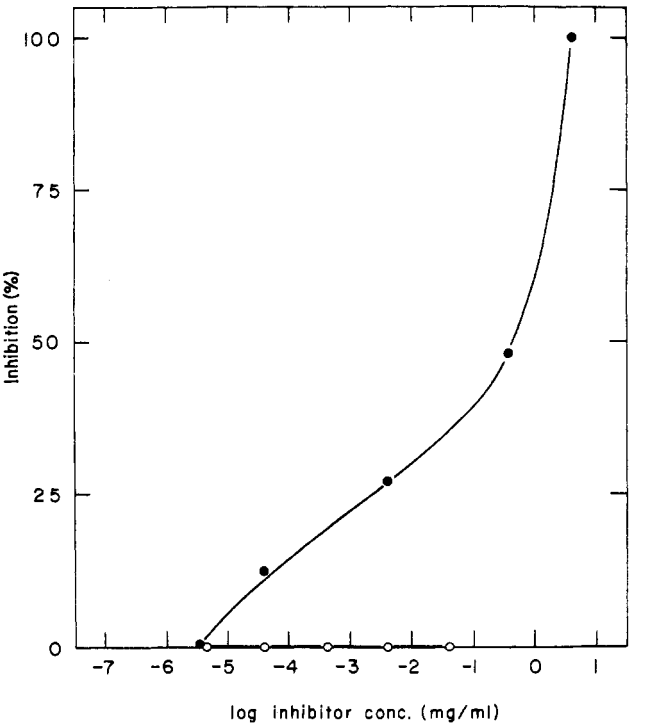

FIGURE 2: Inhibition of the inactivation of (Pro-Gly-Pro)$_n$–RNase–bacteriophage T4 by rabbit anti-(Pro-Gly-Pro)$_n$–ovalbumin serum with RCM *Ascaris* collagen (●) and guinea pig skin collagen (○).

Cross-reaction of (Pro-Gly-Pro)$_n$ with the various natural collagens tested, including rat tail tendon collagen and *Ascaris* cuticle collagen, was demonstrated in guinea pigs at the cellular level much before any antibody formation was detected. Whereas the delayed-type sensitivity became weaker approximately a month after immunization, the intensity of the immediate reactions increased with time. The early manifestation of the immune response to (Pro-Gly-Pro)$_n$ in cross-reactions with natural collagens at the cellular level without eliciting the formation of cross-reacting antibodies is not unique. Similar findings were described for lysozyme and α-lactalbumin (Maron *et al.*, 1972), lysozyme and its reduced and *S*-carboxymethylated derivative (Thompson *et al.*, 1972), flagellin and its acetoacetylated derivatives (Parish, 1971), and also for collagen systems (Michaeli *et al.*, 1972; Adelmann

TABLE IV: Inhibition of the Inactivation of (Pro-Gly-Pro)$_n$–RNase–Bacteriophage T4 with Anti-*Ascaris* Cuticle Collagen by Means of Collagen-like Polyhexapeptides.

Inhibitor	Mol Wt	Inhibitor Concn (M) Needed for 50% Inhibn
(Gly-Pro-Pro-Gly-Pro-Pro)$_n$[a]	1900	2.5×10^{-8}
(Gly-Ala-Pro-Gly-Pro-Pro)$_n$	2800	1.6×10^{-7}
(Gly-Pro-Ala-Gly-Pro-Pro)$_n$	2600	2.5×10^{-7}
(Gly-Ala-Ala-Gly-Pro-Pro)$_n$	2320	5.6×10^{-6}
(Gly-Ala-Pro-Gly-Pro-Ala)$_n$	2400	2.5×10^{-5}

[a] This is the ordered polytripeptide (Pro-Gly-Pro)$_n$. The following polyhexapeptides were obtained by polymerizing respectively the hexapeptides Ala-Pro-Gly-Pro-Pro-Gly, Pro-Ala-Gly-Pro-Pro-Gly, Ala-Ala-Gly-Pro-Pro-Gly, and Ala-Pro-Gly-Pro-Ala-Gly.

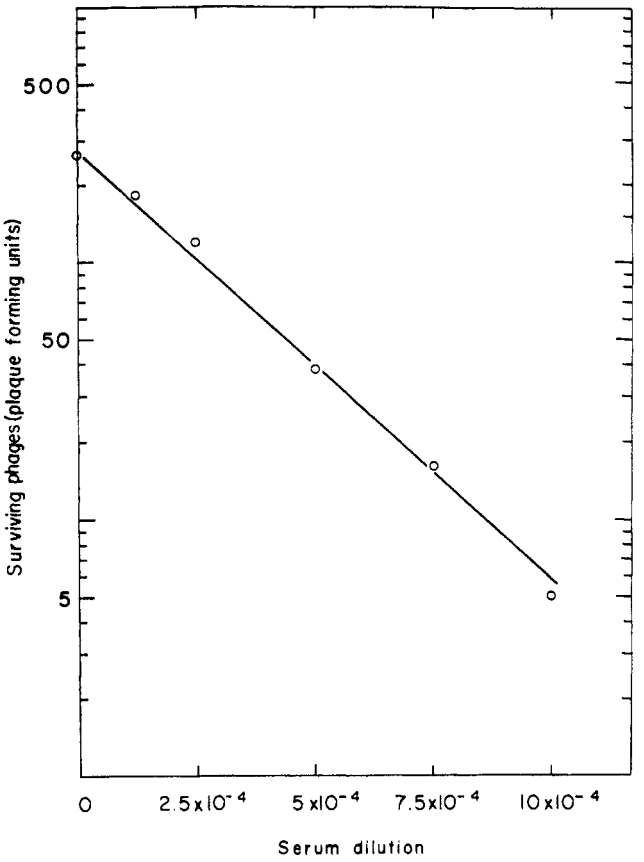

FIGURE 3: Inactivation of (Pro-Gly-Pro)$_n$–RNase–bacteriophage T4 conjugate by rabbit anti-*Ascaris* collagen serum.

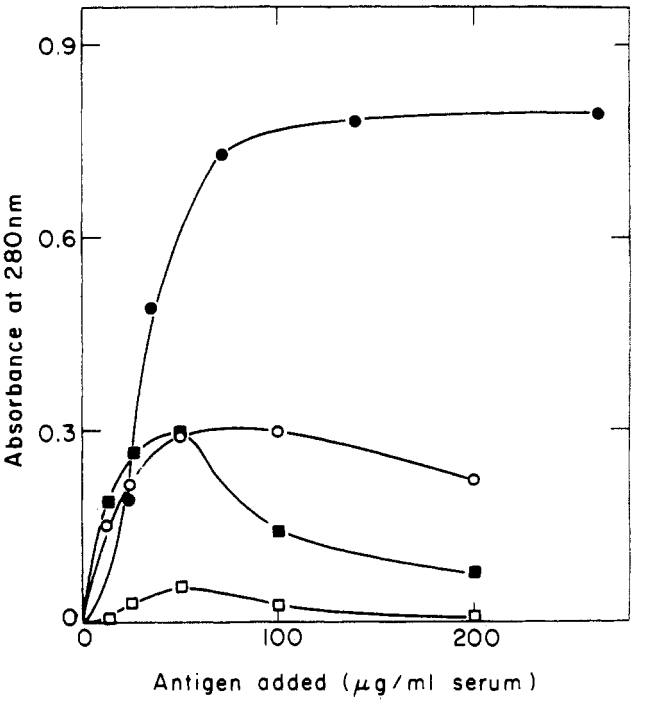

FIGURE 4: Precipitin reactions of rabbit antisera to *Ascaris* collagen and to reduced and carboxymethylated (RCM) *Ascaris* collagen. The homologous reaction of *Ascaris* collagen–anti-*Ascaris* collagen (●); cross-reaction between (Pro-Gly-Pro)$_n$–RNase and anti-*Ascaris* collagen (○); the homologous reaction of RCM *Ascaris* collagen–anti-RCM *Ascaris* collagen (■); cross-reaction between (Pro-Gly-Pro)$_n$–RNase and anti-RCM *Ascaris* collagen (□).

et al., 1972). These differences may be explained by either low initial titers of homologous antibodies or by their low affinity to the antigen. On the other hand, it is also possible that the recognition of different determinants within the same molecule is responsible for the different manifestations of the immune response.

As seen in Tables I and II, when guinea pigs were immunized with (Pro-Gly-Pro)$_n$, positive delayed skin reactions were obtained even with guinea pig skin collagen. Moreover, antibodies produced in guinea pigs cross-reacted with guinea pig collagen, as initially observed by Borek *et al.* (1969). Thus, it would seem that an autoimmune phenomenon was induced by means of a synthetic polypeptide antigen. Autoimmune diseases may be accompanied by a decrease in complement level, due to the fixation of complement to animal's own tissues as a result of their reaction with antibodies. No such decrease was observed when complement levels were measured in normal and immunized guinea pigs. Nevertheless, the possible autoimmune effects described here should be investigated in more detail.

The extent of cross-reactions between the oligomers of Pro-Gly-Pro used as immunogens, and the natural collagens used for testing, appeared to be dependent on the oligopeptide size both at the cellular and the humoral level (Table II). These results are in agreement with a previous study (Engel *et al.*, 1966) showing that the stability of the helical collagen conformation of (Pro-Gly-Pro)$_n$ increases with the molecular weight of the polymer.

Immunization with random copolymers of glycine and proline and/or hydroxyproline in different combinations did not induce any cross-reaction with native collagens (Jasin and Glynn, 1965a,b). Similarly, immunization with the random copolymer (Pro^{66},Gly^{34})$_n$ did not lead to any cross-reaction with the different natural collagens (Table I). Thus, the cross-reactions between natural collagens and the ordered polypeptide (Pro-Gly-Pro)$_n$, and the lack of such cross-reactions with the random polymer containing L-proline and glycine in a similar residue molar ratio, strongly suggest that the immune response toward (Pro-Gly-Pro)$_n$, whether cellular or humoral, is due to conformational determinants within this ordered polymer. These results would imply that the specificity of the immune response is controlled entirely by the higher order structure of the collagen-like immunogen, and confirm the crucial role of steric conformation in the immune response (Sela *et al.*, 1967).

Since (Pro-Gly-Pro)$_n$ possesses conformation similar to that of collagens it is possible that upon immunization with the ordered polymer, a large portion of the antibodies which may cross-react with native collagen would be absorbed by autologous protein. The application of very sensitive immunological techniques, such as the inactivation of chemically modified bacteriophage (Haimovich *et al.*, 1970a,b), enabled us to demonstrate cross-reactions between the synthetic collagen-like ordered polymer (Pro-Gly-Pro)$_n$ and natural collagens. Timpl *et al.* (1971) reported recently that antibodies to rabbit collagen did not react with either (Gly-Pro-Pro)$_n$ or (Gly-Pro-Ala)$_n$. The latter polymer is also known to be a collagen model (Heidemann and Bernhardt, 1968). This lack of cross-reactions may be due either to the lack of sensitivity of the assay, or to a large proportion of antisequential antibodies in the rabbit antisera.

Cross-reactions were reported between the octapeptide Gly-Pro-Gly-Pro-Pro-Gly-Ala-Lys and collagen (Kettman

et al., 1967) as well as gelatin (E. Benjamini, personal communication). On the other hand, we were not able to show any cross-reactivity between an antiguinea pig gelatin antiserum and $(Pro\text{-}Gly\text{-}Pro)_n$ as tested with $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–bacteriophage T4. The cross-reactions of the octapeptide with collagen and gelatin may thus be due to common sequential determinants.

The basic structure of collagens is common due to the highly repetitive sequence of the Gly-Pro (or Hyp)-X triplets. Different contents and distributions of imino and amino acids along the chains of the molecule would contribute to the species specific differences among collagens. The correlation between the total pyrrolidine content and several properties of some invertebrate collagens was systematically surveyed (Josse and Harrington, 1964). The distribution of pyrrolidine residues in triplets of various collagens of known composition was estimated statistically. From their calculated frequencies of contiguous pyrrolidines it can be seen that the highest incidence of dipyrrolidines appeared in *Ascaris* cuticle collagen. In this collagen the calculated values correspond mainly to prolines, according to known amino acid composition. This unique feature of *Ascaris* collagen as well as its far phylogenetical distance from the other collagens tested, seems to contribute to a great extent to the high immunogenicity (Fuchs and Harrington, 1970) and the cross-reactivity of this collagen with $(Pro\text{-}Gly\text{-}Pro)_n$ both at humoral and cellular level. Cross-reactions between *Ascaris* collagen and $(Pro\text{-}Gly\text{-}Pro)_n$ were observed by various immunological techniques, such as precipitin reaction (Figures 1 and 4), passive cutaneous anaphylaxis (Table III), hemagglutination and modified bacteriophage inactivation, and inhibition (Figures 2, 3, and 5).

The specificity of the humoral cross-reaction between RCM *Ascaris* collagen and the ordered polymer was tested by pca reactions with antisera to *Ascaris* collagen and to $(Pro\text{-}Gly\text{-}Pro)_n$–ovalbumin conjugate, before and after immunospecific adsorption on $(Pro\text{-}Gly\text{-}Pro)_n$–Sepharose (Table III). It was clearly demonstrated that the antiserum to *Ascaris* collagen contains antibodies of two distinct specificities, one to common collagen conformation, and the other, species specific. The localization of the antigenic determinants along the collagen molecule is a subject which is still extensively studied and described in numerous papers. Steffen *et al.* (1968) related the antigenic sites in the middle region of the collagen molecule to sequences of the type Gly-Pro-X in which X mainly denotes proline, hydroxyproline, or alanine. Davison *et al.* (1967) suggested that the main antigenic sites of collagen reside in peptide chains protruding from the triple-helical body of the molecule. These peptides can be split off by proteases under conditions which leave the triple-helix conformation intact. Other reports describing the use of proteases in order to identify antigenic determinants have appeared in recent literature (Timpl *et al.*, 1970; Steffen *et al.*, 1968, 1970; Furthmayr *et al.*, 1971). It seems that the type of the assay as well as the phylogenetic barrier between the source of the collagen and the animals used for immunization should be taken in consideration in the general effort to establish the localization of the antigenic determinants in the collagen molecule.

An additional unique feature of *Ascaris* cuticle collagen is that its triple-helix structure is formed by a reverse folding within a single chain (McBride and Harrington, 1967). This would imply an antiparallel alignment inside the *Ascaris* collagen molecule. The results of Engel *et al.* (1966) suggest that $(Pro\text{-}Gly\text{-}Pro)_n$ in solution is made up of single chains

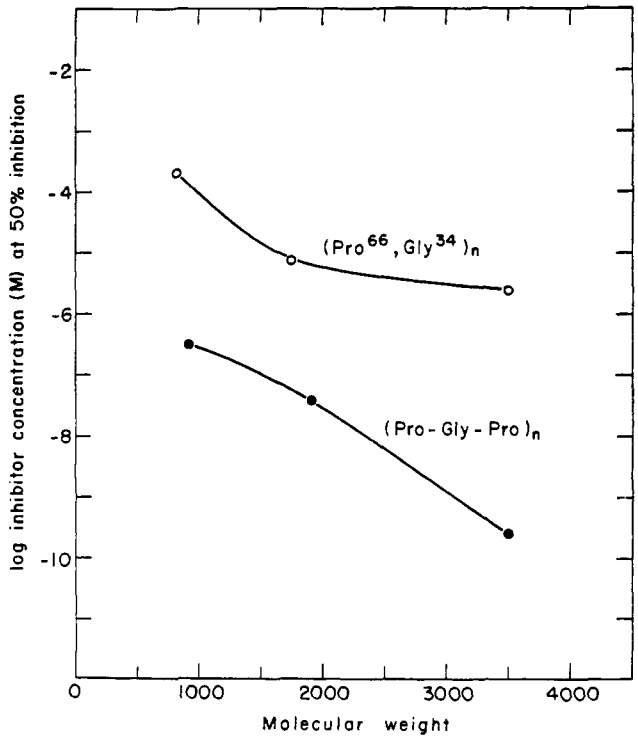

FIGURE 5: Molecular weight dependence of the inhibitory capacity of the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ and the random polymer $(Pro^{66},Gly^{34})_n$. The concentrations of inhibitor required for 50% inhibition of the inactivation of $(Pro\text{-}Gly\text{-}Pro)_n$–RNase–bacteriophage T4 by anti-*Ascaris* collagen serum are plotted.

which show triple-stranded collagen structures; this would also imply that the chains fold back on themselves. On the other hand, other measurements in the solid state and in aqueous solution suggest that chains of the ordered polymer lie parallel. The strong cross-reactions between *Ascaris* collagen and $(Pro\text{-}Gly\text{-}Pro)_n$ should be taken in account in attempts to draw a definite conclusion about the structure of the $(Pro\text{-}Gly\text{-}Pro)_n$ molecules in solution.

The specificity of antiserum to *Ascaris* collagen was studied by cross-reactions with four polyhexapeptides containing proline, glycine, and alanine in different sequences (Table IV). These synthetic ordered polymers were also shown to be valid collagen models (Segal, 1969; Segal *et al.*, 1969). The extent of cross-reactions between the four polyhexapeptides tested and anti-*Ascaris* collagen serum increased markedly as the sequence of the respective polyhexapeptide was closer to Gly-Pro-Pro-Gly-Pro-Pro and thus also more similar to *Ascaris* collagen. These results confirm the calculated values for incidence of dipyrrolidines in the *Ascaris* collagen molecule (Josse and Harrington, 1964).

In view of the results presented in this paper it would be of interest to find out whether it is possible to follow biological functions of collagen with antibodies to the synthetic collagen model $(Pro\text{-}Gly\text{-}Pro)_n$ by virtue of their cross-reactivity with natural common collagen determinants. Indeed, cytotoxic effect of these antibodies on rat fibroblasts in the presence of complement was recently observed (Maoz *et al.*, 1973b).

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