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The Antigenicity of Native and Tyrosylated Neutral-Salt-Soluble Rat Collagen

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Summary. Neutral-salt-soluble collagen was extracted from rat skin and purified by repeated precipitation, resolution and dialysis. Part was reacted with N-carboxy-tyrosine anhydride and a collagen derivative containing 2·6 per cent tyrosine was recovered. Enrichment with tyrosine did not alter the optical rotation, denaturation temperature or electrophoretic mobility of the collagen.

The antigenic properties of native and tyrosylated rat collagen were studied in rabbits and guinea-pigs by micro-complement fixation, tanned cell agglutination and agglutination-inhibition, passive cutaneous anaphylaxis and immediate and delayed skin hypersensitivity. The antigenicity of native collagen was demonstrated. Enrichment with a limited amount of tyrosine enhanced its antigenicity without altering its antigenic specificity and permitted a detailed analysis of the overall specificity of the immunological reaction. Use of the products of controlled degradation of collagen in the immunoassay systems implicitly defined the collagen molecule as responsible for the immune reaction. Collagenase-digestion products still possessed antigenic capacity.

INTRODUCTION

The weak antigenicity of collagen is well known. Some investigators failed to detect its antigenicity (Waksman and Mason, 1949; Peacock and Petty, 1960; Grillo and Gross, 1962) while earlier workers did not define serological specificity (Loiseleur and Urbain, 1930). There is evidence, however, that collagen possesses some antigenic capacity. Thus, in immunized animals, Watson, Rothbard and Vanamee (1954), Paz, Davidson, Gomez and Mancini (1963) and Schmitt, Levine, Drake, Rubin, Pfahl and Davison (1964) reported having found complement fixing antibodies; Steffen, Timpl and Wolff (1964) agglutinating antibodies, and O'Dell (1965) precipitating antibodies. Jasin and Glynn (1965b) demonstrated immediate and delayed skin hypersensitivity in guinea-pigs immunized with native or acetylated collagen.

Successful immunization usually followed the administration of soluble antigen but antibody titres were often low even after long periods of immunization. The weak antigenicity of collagen has been related to low aromatic and sulphur content, high imino acid content, ability to resist the degradative powers of the usual proteolytic enzymes and failure of recognition mechanisms in closely related species.

Gelatin, the denaturation product of collagen, is also a weak antigen. By adding tyrosyl residues to this randomly coiled protein, Arnon and Sela (1960) were able to enhance

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its antigenicity but the amount of tyrosine added was a limiting factor in serological specificity.

This investigation sought to enhance the antigenicity of collagen by the enrichment of the molecule with a limited amount of tyrosine, and to define collagen–anti-collagen specificity by appropriate modifications of the molecule.

MATERIALS AND METHODS

Antigens

(1) *Neutral-salt-soluble collagen (NSC).* Under cold-room conditions (2–4°) NSC was extracted from comminuted rat skin with a neutral salt solution (0·45 M NaCl, 0·02 M phosphate buffer, pH 7·2) and purified by precipitation procedures employing sodium chloride (Jackson and Fessler, 1955) or ethanol (Gross, 1958). Purification was monitored spectrophotometrically (Fessler, 1960). The purified collagen was stored in the cold as a solution in acetic acid. When desired, aliquots were transferred to a neutral medium by dialysis and clarified by centrifugation at 100,000 ***g*** for 2 hours. The pure material contained 0·6 per cent tyrosine and was completely soluble in 5 per cent trichloroacetic acid at 45°.

(2) *Tyrosylated neutral-salt-soluble collagen (TNSC).* The method of tyrosylation was that which Arnon and Sela (1960) applied to gelatin except that dimethylformamide was used as a solvent for the tyrosylating agent. NSC was reacted at 2° for 24 hours with N-carboxytyrosine anhydride (Cyclo Chemical Corporation, Los Angeles) in a saline–dimethylformamide mixture to give a tyrosine enrichment of about 2 per cent. At completion the collagen derivative was precipitated with 20 per cent NaCl and washed several times with 50 per cent acetone to eliminate any polytyrosine which may have been synthesized. The washed precipitate was dissolved in 0·1 M acetic acid, dialysed exhaustively against this solvent to remove remaining free tyrosine and stored at 2°. The modified collagen contained 2·6 per cent tyrosine. Tyrosylation decreased slightly the solubility of the collagen in neutral salt solution and in 5 per cent trichloroacetic acid at 45°, but optical rotation, denaturation temperature and electrophoretic mobility remained unchanged (Kirrane, 1967).

(3) *Denatured NSC and TNSC.* Neutral salt solutions or acetic acid solutions of NSC and TNSC were dialysed against sodium acetate buffer (0·1 M, pH 4·1) for 48 hours and denatured by heating at 45° for 30 minutes. The kinetics of denaturation of native and tyrosylated collagen were monitored by polarimetry. Identical phase transition profiles were obtained for NSC and TNSC.

(4) *Collagenase-digested NSC and TNSC.* The collagens were digested with collagenase (Code CLSP, Worthington Biochemicals, New Jersey) as described by Gallop, Seifter and Meilman (1957). Collagenolysis was followed by measuring liberated amino groups using trinitrophenylsulfonate (Satake, Okuyama, Ohashi and Shinoda, 1960).

Immunization procedure

Rabbits received injections into the footpads of 1 mg of NSC or TNSC in complete Freund's adjuvant (Difco). Two weeks later they received a similar injection intradermally between the scapulae. Thereafter at fortnightly intervals for 6 months they received 1 mg of soluble antigen intravenously. Blood was obtained prior to each injection and the serum stored at −20° without preservative. All sera were inactivated at 56° for 30 minutes before use.

Guinea-pigs received injections into the footpads of 1 mg of NSC or TNSC in complete Freund's adjuvant. Some guinea-pigs were skin-tested at 2 weeks. The remainder were given a second injection similar to the first but intradermally between the scapulae and were skin-tested at 4 weeks.

In vitro *tests for antibody*

Micro-complement (C′) fixation was done according to Wasserman and Levine (1961) but lysis was determined by the method of Rosenberg and Tachibana (1962) using ^{51}Cr-labelled erythrocytes. The final reaction volume was 0·5 ml. The experiment was prepared in an ice bath and fixation was allowed to proceed at 2° for 18 hours. Twenty per cent C′ fixation or greater was considered indicative of a C′ fixing antigen–antibody reaction. Guinea-pig serum served as C′ source. Small aliquots were snap frozen in a dry-ice–acetone mixture and stored in sealed ampoules in a dry-ice chest. A fresh sample was removed for each day's testing.

Tanned-cell agglutination and agglutination-inhibition was done according to Stavitsky (1954) except that isotonic veronal buffered saline, pH 7·3, incorporating 0·1 per cent bovine serum albumin was used as diluent throughout. Guinea-pig red cells were used for tanning and coating and a collagen concentration of 0·01 per cent proved optimal for sensitization. Non-immune sera and uncoated tanned red cells were used as controls with every experiment. Agglutination-inhibition tests were done by incubating a constant amount of antibody (×60 of titre) with a doubling dilution series (100–0·05 μg) of the inhibitor for 1 hour prior to the addition of the tanned sensitized red cells. With one exception all experiments were conducted at 2°. Inhibition with denatured collagen was done at room temperature to preclude the possibility of regeneration of native collagen molecules.

Capillary precipitation and Ouchterlony double diffusion techniques were employed to detect precipitating antibody. To enhance the sensitivity of the Ouchterlony method the reactant wells were replenished or diffusion of antigen was allowed to take place for a time before antiserum was added to the adjacent wells.

In vivo *tests for antibody and delayed hypersensitivity*

Active cutaneous anaphylaxis in guinea-pigs was done according to Jasin and Glynn (1965a) using 50, 10 and 1 μg of the test antigens. Passive cutaneous anaphylaxis in guinea-pigs was done by the Ovary technique (Ovary, 1958), using undiluted antiserum and dilutions of 1:10 and 1:100.

Other methods

Collagen solutions for hydroxyproline analysis were hydrolysed by heating in sealed tubes with 6 N HCl for 24 hours at 110°. The hydrolysates were evaporated to dryness and the residues were dissolved in a known volume of water. The method of Bergman and Loxley (1963) was adapted to the determination of 1–15 μg of hydroxyproline.

Tyrosine in collagen was determined by the procedure of Beaven and Holiday (1952).

The subunit composition of thermally denatured collagen was ascertained by electrophoresis in polyacrylamide gels according to Nagai, Gross and Piez (1964). In fresh material there were four well defined bands in two distinct sets, one set in the α region and one set in the β region and no demonstrable γ component. Identical electropherograms were obtained for NSC and TNSC.

RESULTS

COMPLEMENT FIXATION

Rabbit immune sera contained C′ fixing antibody against the two forms of rat collagen used for immunization. Anti-TNSC activity was evident after two injections and antisera were obtained in the 2nd month which fixed 65 per cent of the complement at dilutions of 1:200 (Fig. 1). In contrast, only a weak immune response was provoked by native collagen. There was no demonstrable anti-NSC activity during the first 4 months of immunization. Thereafter antisera were obtained which fixed 30 per cent of the complement at dilutions of 1:70 (Fig. 1). A feature of the immune response to both antigens was that it

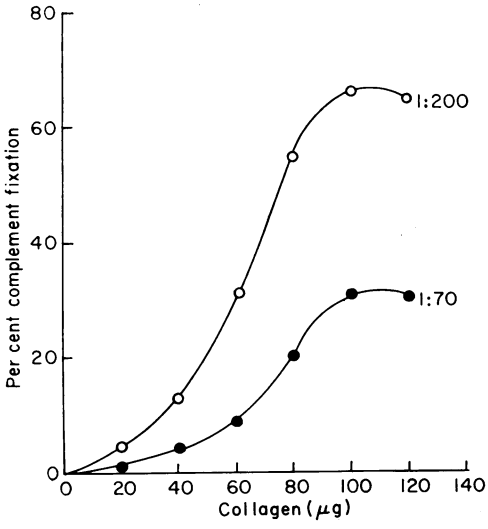

FIG. 1

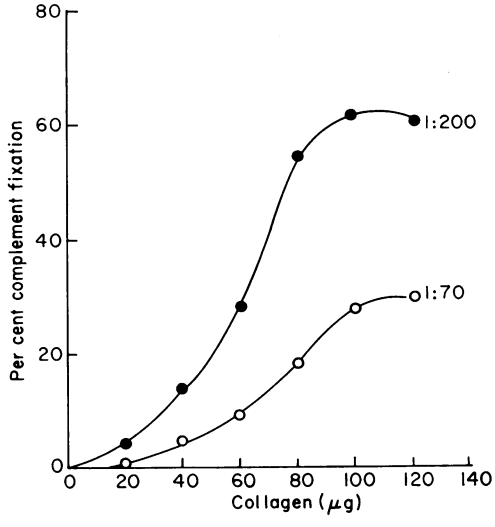

FIG. 2

FIG. 1. Micro-complement fixation by native (●) and tyrosylated (○) rat collagen and the respective antisera diluted as indicated.

FIG. 2. Micro-complement fixation by native (●) and tyrosylated (○) rat collagen and the converse antisera diluted as indicated. Note superimposability of curves with Fig. 1.

was almost maximally established at the outset with little further rise in antibody titre despite repeated immunization. At the constant antibody and complement concentrations employed, specific C′ fixation was maximal in the presence of 100 μg of collagen: antigen excess regions of the curves could not be delineated because higher concentrations of collagen fixed complement non-specifically. Antisera diluted less than 1:50 were also anticomplementary. The pattern of cross-reactivity between native and tyrosylated collagen was studied. With a given antiserum and either antigen the C′ fixation curves obtained were consistently superimposable (within the 10 per cent error of the method) (Fig. 2). This and other evidence (see later) indicates that serological specificity was retained by the carrier protein.

Efforts to obtain evidence of collagen specificity in this system using the degraded forms of collagen were unsuccessful. Denatured and digested collagens were anti-complementary at the concentrations used in the test and precluded accurate measurement of specific C′ fixation. This could not be overcome by using an excess of C′ since two C′

fixing systems cannot be titrated simultaneously because of the unequal rates of consumption of the different components of complement (Heidelberger, 1964). In the experiment with digested collagen the reaction products were not separated, but collagenase used as a control did not fix complement.

TANNED CELL AGGLUTINATION

Antisera against NSC and TNSC whose C′ fixing ability had been accurately determined were tested for their ability to agglutinate tanned cells coated with NSC. Anti-TNSC sera frequently agglutinated at 1:6400 dilution but titres for anti-NSC sera rarely exceeded 1:100. Agglutinating activity was inhibited by prior incubation of the antiserum with microgram quantities of collagen and both antigens were equally effective inhibitors of either system, i.e. NSC inhibited the agglutinating activity of antisera prepared against TNSC and conversely. The inhibitory efficiency of the degraded forms of collagen was ascertained. Denatured NSC (or TNSC) was a better inhibitor of agglutination than the parent compound and the ability of digested NSC (or TNSC) to inhibit agglutination, while diminished, was by no means abolished. Collagenase alone in equivalent amounts was not inhibitory. The results of inhibition experiments are summarized in Table 1.

TABLE 1

INHIBITION OF AGGLUTINATION OF TANNED GUINEA-PIG ERYTHROCYTES COATED WITH NATIVE COLLAGEN BY SERIAL TWO-FOLD DILUTIONS OF DIFFERENT COLLAGEN PREPARATIONS

Coating antigen	Inhibiting antigen	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Native collagen	Native collagen	−	−	−	−	−	−	−	−	−	+	++	+++	+++	+++
	Tyrosylated collagen	−	−	−	−	−	−	−	−	−	+	++	+++	+++	+++
	Denatured collagen	−	−	−	−	−	−	−	−	−	−	−	−	+	++
	Digested collagen	−	−	−	−	−	+	+	++	++	++	+++	+++	+++	+++

Tube No. 1 contains 100 μg of inhibitor. The antiserum diluted to ×60 of titre was made against tyrosylated collagen. − = No agglutination; + = minimal agglutination; ++ = moderate agglutination; +++ = complete agglutination.

PRECIPITATION

Antiserum of high C′ fixing and agglutinating activity did not contain precipitating antibody by capillary precipitation or double diffusion in agar gel. Prediffusion of antigen and/or replenishment of reactant wells during the course of a test was without effect.

CUTANEOUS ANAPHYLAXIS

Results obtained in the PCA procedure were equivocal. Negative and non-specific reactions, namely the occurrence of blueing at the test site prior to injection of the antigen, were observed.

The antigenicity and cross-reactivity of NSC and TNSC could be further demonstrated in the sensitized guinea-pigs. Immediate and delayed hypersensitivity reactions were demonstrable after a single immunizing injection of TNSC and the immune response was firmly established after a repeat injection (Table 2). There was no response to a single

TABLE 2

CUTANEOUS ANAPHYLAXIS IN THREE GUINEA-PIGS AFTER PRIMARY IMMUNIZATION AND RE-IMMUNIZATION WITH TYROSYLATED RAT COLLAGEN

Test antigen	Amount (μg)	Immunizing antigen–tyrosylated collagen											
		Primary immunization						Re-immunization					
		Immediate			Delayed			Immediate			Delayed		
		1	2	3	1	2	3	1	2	3	1	2	3
Tyrosylated collagen	50	10	9	7	10	12	10	9	10	11	14	14	16
	10	7	5	5	7	8	7	6	8	6	9	10	12
	1	5	5	–	5	6	5	5	6	–	6	7	7

The results are expressed as the diameter of the reaction site. A diameter less than 5 mm was read as –.

TABLE 3

CUTANEOUS ANAPHYLAXIS IN THREE GUINEA-PIGS AFTER PRIMARY IMMUNIZATION AND RE-IMMUNIZATION WITH NATIVE RAT COLLAGEN

Test antigen	Amount (μg)	Immunizing antigen–native collagen											
		Primary immunization						Re-immunization					
		Immediate			Delayed			Immediate			Delayed		
		1	2	3	1	2	3	1	2	3	1	2	3
Native collagen	50	–	–	–	–	–	–	8	10	10	10	12	12
	10	–	–	–	–	–	–	6	8	7	7	9	8
	1	–	–	–	–	–	–	–	5	5	–	6	6

The results are expressed as the diameter of the reaction site. A diameter less than 5 mm was read as –.

immunizing injection of NSC but reactions closely approximating those against TNSC were seen after a second injection (Table 3). The presence of circulating antibody was confirmed by tanned cell haemagglutination. Sensitized animals responded equally well to challenge with either form of collagen irrespective of which was the immunizing antigen. Delayed reactions were noticeably stronger than immediate reactions and there was a graded response to decreasing amounts of test antigen. Most animals gave a measurable response to 1 μg of collagen. Arthus reactions were not observed. All animals were skin tested with normal rat serum at a dilution of 1 : 100 and some weak positive immediate reactions were recorded. Normal guinea-pigs and guinea-pigs treated with complete Freund's adjuvant alone did not react to the test antigens.

The immunological reactivity of the degraded collagens was studied in this system. Animals immunized with NSC gave equally good immediate and delayed reactions when challenged with NSC or denatured NSC (Table 4). When digested collagen was used as test antigen the immediate reactions were comparable but there was a marked decrease in the intensity of the delayed reaction (Table 4). Collagenase alone at ten-fold greater concentration than that used in the test did not cause any reactions and normal animals did not react to the collagenase digest.

TABLE 4

CUTANEOUS ANAPHYLAXIS IN THREE GUINEA-PIGS IMMUNIZED WITH NATIVE COLLAGEN AND TESTED WITH NATIVE COLLAGEN, DENATURED COLLAGEN AND DIGESTED COLLAGEN

Test antigen	Amount (μg)	Immunizing antigen–native collagen					
		Immediate			Delayed		
		1	2	3	1	2	3
Native collagen	50	9	9	10	10	10	12
	10	6	6	7	8	8	8
	1	–	5	5	6	6	7
Denatured collagen	50	9	10	10	10	14	10
	10	6	6	5	8	10	5
	1	5	5	–	6	5	–
Digested collagen	50	10	9	9	5	6	–
	10	7	6	6	–	–	–
	1	5	–	–	–	–	–

The results are expressed as the diameter of the reaction site. A diameter less than 5 mm was read as –.

DISCUSSION

The above results are in general agreement with current opinion which ascribes weak antigenic properties to collagen. However, enrichment of the molecule with a limited amount of tyrosine had the desired effect of provoking a more vigorous immune response in rabbits and guinea-pigs without altering collagen–anti-collagen specificity. The way in which tyrosine effects an enhancement of the antigenicity of proteins is not known.

In the continued search for anti-collagen antibodies C′ fixation has enjoyed pre-eminence as an assay procedure. Waksman and Mason (1949) and Peacock and Petty (1960), using insoluble collagen for immunization, could not demonstrate antibodies by this technique, but other workers using soluble collagen as antigen reported success. Thus, Watson *et al.* (1954) found low titres of C′ fixing antibody in rabbits immunized with soluble collagen of rat origin. Thermal denaturation did not influence the serological activity of the collagen, but collagenase digestion abolished this property. Paz *et al.* (1963) also by C′ fixation demonstrated low grade antigenicity in rabbits immunized with soluble and insoluble collagens of chick origin, and defined specificity on the basis of absorption studies. More recently Schmitt *et al.* (1964) induced C′ fixing antibodies in rabbits by immunization with soluble calf skin collagen, but the data presented do not permit an assessment of the intensity of the immune response. These investigators assessed specificity by its susceptibility to thermal denaturation and collagenase digestion. In contrast to Watson *et al.* (1954) they found that denatured collagen could no longer fix complement, but they agreed that the serological activity of digested collagen was abolished. There are, therefore, opposing views on the C′ fixing ability of thermally denatured collagen. We could not resolve this question for the reasons outlined, but our other findings favour the view that both thermally denatured and collagenase-digested collagen are immunologically active. It is noteworthy that Schmitt *et al.* (1964), using the Wasserman–Levine micro-C′ fixation procedure, obtained peak fixation of complement with 1 μg of collagen or less. In our hands 100 μg were necessary. The reason for these massive differences in antigen requirements for peak fixation of complement in two closely related collagen–anti-collagen systems is not clear. It may be a reflection of technical procedure or of the

relative potency of the antisera, but in view of the low grade antigenicity of collagen the large amount required for peak fixation in our system may be the result of the specificity of the antibody for only infrequent determinant groups.

Tanned cell testing confirmed and extended the findings of C′ fixation. Here the weak antigenicity of NSC was again evident and the superior antigenicity of TNSC was further substantiated. Moreover, as can be seen from the inhibition studies, serological specificity had not been altered by tyrosylation. The inhibition of the reaction with microgram quantities of collagen and the modifications engendered in the antigen–antibody reaction by thermal denaturation and collagenase digestion provide impressive evidence for specificity. It is unlikely that a contaminant would possess physical properties such that heating at 45° or digestion with collagenase would modify its immunological activity. It could however be anticipated that both manipulations would alter the immunological reactivity of collagen. Such was the case. When the polypeptide chains of collagen were randomly distributed their inhibitory efficiency was increased. Stoller and Levine (1960) had a similar experience with denatured desoxyribonucleic acid in the micro-C′ fixation system and concluded that the enhancement was a function of reactive sites previously hidden in the interior of the folded molecule. The inhibition by collagenase-digested collagen was unequivocal and demonstrates for the first time the immunological reactivity of a collagen which had been digested by a specific protease. The products deriving from collagenase digestion are mostly small peptides of average molecular weight 500. Seifter, Gallop and Meilman (1958) showed that these fragments retain some triple helical structure and emphasized that this was not unexpected since the site of collagenase action does not reside in the secondary bonds which give collagen its folded configuration. Some residual immunological reactivity could, therefore, reasonably be expected after collagenase digestion.

Precipitating antibody was not demonstrated. It may not have been formed or the optimal conditions for its demonstration may not have obtained. Fibrinogen, a viscous elongated molecule with dimensions and molecular weight similar to collagen, behaves anomalously in classical double diffusion systems and steric hindrance has been postulated as the impeding mechanism (Wolf and Walton, 1965). A similar situation may exist in relation to collagen, but it appears from the work of O'Dell (1965) that precipitating antibody to collagen may be obtained with relative ease by crossing phyletic barriers.

The negative and nonspecific PCA reactions are of some interest. The only other account of PCA testing in a collagen–anti-collagen system is that of Paz *et al.* (1963) and they too recorded variable and nonspecific results. Their findings were not discussed. The exact site of fixation of antibody in PCA has not been established, but it is generally believed to be outside the dermal venules. Biozzi, Benacerraf and Halpern (1953) have shown that globular particles greater than 350 Å in diameter cannot pass the endothelial basement membrane. In one dimension collagen is greater than this by almost an order of magnitude, but because of its elongated form it should negotiate the basement membrane barrier if it remains in the monomeric state. But in a PCA test monomeric collagen is dispersed in an environment at 37°, a temperature which is known to be optimal for the formation of higher aggregates *in vitro*. Polymeric forms of collagen grossly disproportionate to basement membrane pore size may form under these conditions, thus pre-empting the possibility for reaction at its intended destination. The blueing frequently observed at the test site prior to injection of the antigen was puzzling, but if guinea-pig and rat collagens are immunologically closely related (Rothbard and Watson, 1965) determinant sites of

guinea-pig skin collagen could react with the antibody made against rat skin collagen. Under such circumstances injections of the dye would reveal a *fait accompli*. PCA appears therefore not to be a suitable procedure for assay of anticollagen antibody.

Further evidence for antigenicity was forthcoming from our studies in active cutaneous anaphylaxis. This was an ideal system in which to study the immunological reactivity of collagen because the antigen and its degradation products could be deposited in the skin of the sensitized animals and visual comparisons of reactivity made. A good immune response with the production of humoral and cell-bound antibody was provoked in guinea-pigs with a single injection of tyrosylated collagen, but it is noteworthy that two injections of native collagen provoked an immune response of comparable magnitude. Similarly, Jasin and Glynn (1965b) obtained immediate and delayed reactions in guinea-pigs after one or two injections of calf collagen preparations. In a study of skin hypersensitivity reactions Grillo and Gross (1962) failed to demonstrate active cutaneous anaphylaxis in rabbits immunized with soluble calf collagen. They did however observe that the immunized animals gave delayed reactions to calf plasma and adduced this as evidence of contamination of the stock collagen by plasma protein. We observed weak immediate reactions to rat serum in some animals immunized with the rat collagen preparations, and in view of the formidable task of purifying collagen the possibility mentioned by Grillo and Gross must be conceded. But another interpretation is possible. A hydroxyproline-containing protein has been isolated from rat serum which has many collagen-like properties and is believed to be a circulating form of collagen (Le Roy, Kaplan, Udenfriend and Sjoerdsma, 1964). This macromolecule could be responsible for the observed cross-reaction with rat serum. The responses given by denatured collagen further substantiate our view that disorganization of the helical structure does not diminish the immunological reactivity of collagen. Furthermore, if complement is consumed in immediate anaphylactic reactions (Osler, 1961; Ishizaka, 1963), denatured collagen must be considered capable of fixing complement *in vivo*, thus favouring the view of Rothbard *et al.* (1954) that it can also fix complement *in vitro*. The experiments with digested collagen gave valuable evidence of immunological specificity. The fact that such a clear-cut modification of the immune response was obtained after collagenase action together with the findings in passive haemagglutination clearly illustrates the role of collagen in the genesis of the response. That the immediate reaction was retained after collagenase attack while the delayed reaction was only marginally elicitable is of interest. There is evidence that the determinant site for immediate hypersensitivity constitutes only a small area of an antigen molecule, while that for delayed hypersensitivity is larger and involves amino acid residues extending away from the immediate hypersensitivity area (Gell and Benacerraf, 1961; Jasin and Glynn, 1965a). As already mentioned, the products deriving from collagenase hydrolysis are small peptides which, although capable of mediating the immediate reaction, are too small to give the customary delayed response.

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