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Studies on the Chemical Basis of the Antigenicity of Proteins

3. THE ROLE OF RIGIDITY IN THE ANTIGENICITY OF POLYPEPTIDYL GELATINS*

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An investigation of the antigenicity of various polypeptidyl gelatins (Sela & Arnon, 1960a) showed that the enrichment with tyrosine, tryptophan or phenylalanine converted gelatin into relatively powerful antigens of narrow specificity. The attachment of cysteine to gelatin caused a limited enhancement of antigenicity. Enrichment of gelatin with alanine, lysine or glutamic acid did not increase significantly the antigenic response.

From a comparison of the antigenic specificity of several polytyrosyl gelatins (Arnon & Sela, 1960) it was concluded that the serological specificity changes strongly as a function of the tyrosine content. Thus gelatin cross-precipitated with antibodies to a derivative with a low tyrosine content, inhibited the homologous reaction of a material with a somewhat higher tyrosine content, but did not interfere at all with the serological reaction of a derivative enriched with the largest amount of tyrosine (11%). It seemed of interest to investigate the cross-reaction of the various polypeptidyl gelatins with the antiserum to gelatin. Here, again, the increase in the tyrosine content diminished considerably the extent of precipitation.

In view of the above-mentioned results we

assumed that the enhancement of the antigenicity of gelatin by the attachment of the aromatic α -amino acids and of cysteine was due to an increase in the rigidity of the molecule. According to Haurowitz (1952) a rigid structure of the determinant groups is a prerequisite for antigenicity. Even though the content of sulphhydryl groups in polycysteinyl gelatin did not change significantly during a period of several months, it is possible that disulphide bonds, which would increase the rigidity of the macromolecule, are formed *in vivo*. It was therefore of interest to investigate the antigenicity of polyseryl gelatin. Serine differs from cysteine only by the oxygen which replaces sulphur, and thus no cross-linking bridges may be formed as a result of the attachment of serine to gelatin. As expected, the attachment of serine did not convert gelatin into a more powerful antigen.

In order to elucidate the role of the aromatic character of the aromatic amino acids attached, in the enhancement of the antigenicity, we have now investigated the immunological activity of polycyclohexylalanyl gelatins. This paper presents evidence that the attachment of amino acids containing the fully saturated cyclohexane rings caused a strong increase in the antigenicity, as compared with the original gelatin.

* Part 2: Arnon & Sela (1960).

It seems thus that areas of rigidity are a necessary prerequisite for the enhancement of the antigenicity of gelatin. The question arises: what are the rigid sites responsible for the antigenicity of the unmodified gelatin molecule? Gelatin contains very few aromatic acids. It has a high percentage of glycine, which interferes with the stability of an α -helical structure. On the other hand, the high content of proline and hydroxyproline in this heat-denatured protein might still contribute towards helical areas of the type found in collagen (Ramachandran & Kartha, 1954; Rich & Crick, 1955) and in poly-L-proline (Cowan & McGavin, 1955; Harrington & Sela, 1958; Steinberg, Harrington, Berger, Sela & Katchalski, 1960). We have therefore attempted to inhibit the specific gelatin-antigelatin precipitin reaction by means of various copolymers containing proline or hydroxyproline or both. The polymers tested had no influence on the homologous precipitin reaction.

EXPERIMENTAL

Materials

The names of amino acids are abbreviated as in Brand & Edsall (1947) and in Sanger (1952) (see Sela & Arnon, 1960a). *cyclo*Hexylalanine is abbreviated as Cha. The samples of gelatin, egg albumin, the polypeptidyl gelatins pAlaGel, pGluGel, pLysGel, pPheGel and pCysGel, the polytyrosyl egg albumin, as well as a copolymer of L-glutamic acid and L-phenylalanine in a residue molar ratio of 1:1, were described by Sela & Arnon (1960a). The polytyrosyl gelatins (pTyrGelA, pTyrGelB, pTyrGelC) were described by Arnon & Sela (1960).

Poly-L-serine [number-average degree of polymerization (n 30)] was a gift from Mr Y. Lapidot. Poly-L-proline (n 170) was obtained from Mr I. Z. Steinberg. Poly-L-hydroxyproline (n 95), a copolymer of L-proline and glycine (in a residue molar ratio of 1:3, n 30), a copolymer of L-proline and sarcosine (in a residue molar ratio of 1:1, n 50) and a copolymer of L-proline, L-hydroxyproline and glycine (in a residue molar ratio of 1:1:1, n 60) were gifts from Dr J. Kurtz. The amino acid sequence in the above copolymers is random. A copolymer of L-ProGlyGly (obtained by the polymerization of a tripeptide derivative, number-average molecular weight 1200) was a gift from Mr Y. Wolman. A copolymer of L-proline, L-glutamic acid and glycine (in a residue molar ratio of 1:1:2) was prepared by the copolymerization in pyridine of *N*-carboxy-L-proline anhydride, γ -benzyl-*N*-carboxy-L-glutamate anhydride and *N*-carboxyglycine anhydride, followed by debenzoylation of the polymerization product with anhydrous hydrogen bromide.

Polypeptidyl proteins

Poly-L-cyclohexylalanyl gelatin A (pChaGelA), *poly-L-cyclohexylalanyl gelatin B* (pChaGelB) and *poly-L-cyclohexylalanyl egg albumin*. These were prepared, analogously to other polypeptidyl gelatins and egg albumins (Sela, 1954; Sela & Arnon, 1960a; Arnon & Sela, 1960), by the reaction of *N*-carboxy-L-cyclohexylalanine anhydride with

gelatin (Sela & Arnon, 1960b). pChaGelA contained 4.4% of *cyclo*hexylalanine residues. pChaGelB contained 10.9% of *cyclo*hexylalanine residues. Analysis of the dinitrophenylated pChaGelB showed that in this substance 23 moles of distinct poly-L-*cyclo*hexylalanine chains were attached on the average per 100 000 g. of gelatin (Sela & Arnon, 1960b). The average number of *cyclo*hexylalanine residues per peptide chain was, in this case, 3.5. Poly-L-*cyclo*hexylalanyl egg albumin contained 9.7% of *cyclo*hexylalanine residues. A polypeptidyl gelatin enriched with both L-glutamic acid and L-*cyclo*hexylalanine (pGluChaGel) was also prepared (Sela & Arnon, 1960b). It contained 19.3% of glutamic acid residues (original gelatin contains 10.2% of glutamic acid residues) and 18.3% of *cyclo*hexylalanine residues. A copolymer of L-*cyclo*hexylalanine and L-glutamic acid, in a residue molar ratio of 1:1 (n 50), was prepared by the copolymerization of *N*-carboxy-L-*cyclo*hexylalanine anhydride and γ -benzyl-*N*-carboxy-L-glutamate anhydride, followed by the debenzoylation of the polymerization product with anhydrous hydrogen bromide in acetic acid (Sela & Arnon, 1960b).

Poly-L-prolyl gelatin (pProGel). This was prepared by adding 2.5 m-moles of *N*-carboxy-L-proline anhydride (Kurtz, Fasman, Berger & Katchalski, 1958) in dioxan/g. of gelatin in 0.05M-phosphate buffer, pH 7, according to the method of synthesis of polypeptidyl gelatins (Sela & Arnon, 1960a). The water-soluble reaction product was dialysed against distilled water at 2° for 3 days and freeze-dried. The material contained 25.3% of proline residues, determined by the method of Troll & Lindsley (1955) as modified by Sarid, Berger & Katchalski (1959) (the original gelatin contained 13.1% of proline residues).

Poly-L-seryl gelatin (pSerGelA). This compound was prepared by adding 5 m-moles of *O*-acetyl-*N*-carboxy-L-serine anhydride (J. Kurtz, unpublished work)/g. of gelatin. After 3 days at 2° the reaction mixture was brought to pH 13 with 2M-sodium hydroxide and maintained for 2 hr. at room temperature. The clear solution was dialysed against distilled water at 2° for 3 days and freeze-dried. The material contained 10.2% of serine residues, as determined by ninhydrin colorimetry (Kay, Harris & Entenman, 1956) after chromatography in pyridine-water (65:35, v/v) of the acid hydrolysate, as well as 0.6% of acetyl groups. Commercial bone gelatin contains 3.1% of serine residues (Eastoe, 1955).

Methods

Immunization procedure. The antigens used were incorporated in a water-in-oil adjuvant mixture, as described by Sela & Arnon (1960a). Ready prepared adjuvants were obtained from Difco Laboratories, Detroit 1, Mich., U.S.A. Each material was injected into two rabbits. After pre-immunization bleedings, the antigen was injected into the thighs of the hind-legs of the animals. Four injections of 0.5 ml. of the adjuvant mixture were given, at intervals of 14 days. The animals were bled weekly by cardiac puncture, 2–5 weeks after the last injection. Thiomersal was added to all sera to a concentration of 0.01%.

Quantitative precipitin studies. No precipitation occurred when any of the materials tested were added to the pre-immunization sera. In preliminary tests qualitative precipitin reactions were carried out on antisera of individual rabbits. In all experiments the antisera to a particular

antigen behaved similarly. Thereafter such antisera were pooled before quantitative studies. The quantitative precipitin tests were performed by the usual techniques; when no precipitation occurred, the more refined technique of Maurer (1954) was used. Both methods have been described by Sela & Arnon (1960a).

Inhibition studies. Substances checked as possible inhibitors of the precipitin reactions were added at various concentrations, in a volume of 0.1 ml., to 0.2 ml. of serum. After an incubation of 30 min. at 37° an amount of antigen corresponding to the optimum precipitation was added, and the mixture was incubated at 37° for 1 hr. and then placed in the cold room for 24 hr.

Hydroxyproline. This was determined in the hydrolysates (6N-hydrochloric acid, 105°, 24 hr.) of the antigen-antibody precipitates, as well as in the hydrolysates of the gelatin derivatives themselves, by the method of Neuman & Logan (1950). Commercial bone gelatin contains 11.5% of hydroxyproline residues (Eastoe, 1955).

Spectrophotometric measurements. These were made on a Beckman model DU spectrophotometer, at approx. 25°, with quartz cells of 1 cm. light path, with a capacity of 1 ml.

RESULTS

Polycyclohexylalanyl gelatins

Two poly-L-cyclohexylalanyl gelatins, containing 4.4 and 10.9% of cyclohexylalanine residues respectively, were tested for an immunological response in rabbits. No precipitation occurred when solutions of these materials were added to pre-immunization sera. The antisera were subjected to the precipitin test. Positive reactions were obtained in both cases. Table 1 gives the analysis of the precipitates from the antisera tested with the homologous antigens. The amount of antigen in the precipitates was determined from the hydroxyproline content of the acid hydrolysates. As cyclohexylalanine does not absorb light in the ultra-violet region, the antibody content of the precipitates was obtained directly from the extinction of the neutralized solutions at 280 m μ .

Fig. 1. Extinction of neutral solutions of precipitates obtained by the addition of poly-L-cyclohexylalanyl gelatin A (O) and poly-L-cyclohexylalanyl gelatin B (●) to the antiserum to poly-L-cyclohexylalanyl gelatin A, as a function of the amount of precipitant.

Fig. 2. Extinction of neutral solutions of precipitates obtained by the addition of poly-L-cyclohexylalanyl gelatin B (O) and poly-L-cyclohexylalanyl gelatin A (●) to the antiserum to poly-L-cyclohexylalanyl gelatin B, as a function of the amount of precipitant.

Table 1. Composition of precipitates of the systems pChaGelA-anti-pChaGelA and pChaGelB-anti-pChaGelB

Antigen	Antigen added (μ g./ml. of serum)	Antigen precipitated (μ g.)	Antigen precipitated (%)	Antibody precipitated (μ g.)	Antibody/antigen in precipitate (w/w)
pChaGelA	20	22	100	115	5.2
	50	52	100	224	4.3
	100	72	72	378	5.2
	200	95	47.5	550	5.8
	400	138	34.5	540	3.9
	700	164	23.4	502	3.1
	1200	87	7.3	246	2.8
	2000	31	1.6	41	1.3
pChaGelB	50	49.5	100	196	4.0
	100	74.5	74.5	296	4.0
	200	106.6	53.3	330	3.1
	400	180	45	316	1.8
	700	184	26.2	298	1.6
	1200	252	21	250	1.2

The antibodies to the two polycyclohexylalanyl gelatins are very specific. They could be precipitated by both antigens. The precipitin curves of the homologous and cross-reactions are given in Figs. 1 and 2. Precipitation was obtained also when poly-L-cyclohexylalanyl egg albumin was added to the antisera. No cross-precipitation occurred upon addition of any of the materials listed as possible inhibitors in Table 2, as well as unrelated proteins such as egg albumin, to the antisera.

All those substances that did not precipitate anti-pChaGelA or anti-pChaGelB were tested for their ability to inhibit the specific precipitin reactions. Table 2 summarizes the inhibition experiments. Complete inhibition was observed in both systems only with a gelatin derivative enriched with both cyclohexylalanine and glutamic acid. Gelatin enriched with phenylalanine (pPheGel) is a better inhibitor of the pChaGelB-anti-pChaGelB than of the pChaGelA-anti-pChaGelA reaction. The increased reactivity of pPheGel with antibodies to the derivative richer in cyclohexylalanine points to some steric similarity between the two ring structures. As both gelatin and a linear copolymer containing L-cyclohexylalanine

are effective inhibitors of the two systems, it may be concluded that the active antigenic sites include both cyclohexylalanine and parts of the unmodified gelatin molecule.

Polyseryl gelatin

Antiserum against poly-L-seryl gelatin (pSerGelA) gave no precipitate with the homologous antigen, when subjected to the usual tests. Nor was any precipitation observed when the antiserum was caused to cross-react with gelatin and various polypeptidyl gelatins. The precipitin test was therefore performed in a way similar to that used by Maurer (1954) to demonstrate the antigenicity of unmodified gelatin. Table 3 gives the analyses of the precipitates from the antiserum tested with the homologous antigen.

Antibodies to pSerGelA were precipitated under the above-described conditions also with gelatin, pCysGel and pTyrGelA (Fig. 3). pCysGel was selected because of the chemical similarity between cysteine and serine, and pTyrGelA was tested as a typical unrelated modified gelatin. Poly-L-serine neither precipitated nor inhibited the homologous precipitin reaction.

Table 2. Inhibition tests

In all cases 20, 100 and 1000 μ g. of the inhibiting substance was added to 0.2 ml. of each antiserum, 30 min. before the addition of the homologous antigen. -, Complete inhibition at the highest inhibitor concentration, inhibitory influence even at the lowest concentration; +, partial inhibition at the highest concentration, no effect at the lowest concentration; ++, only slight inhibition at the highest concentration; + + +, no inhibitory effect.

Inhibitor	Effect on pChaGelA system	Effect on pChaGelB system
pGluChaGel	-	-
pPheGel	++	-
pTyrGelA	+++	+++
pTyrGelC	++	++
Gelatin	+	+
Copolymer cyclohexylalanine Glu (1:1)	+	+
Copolymer Phe. Glu (1:1)	++	++

Fig. 3. Extinction of neutral solutions of precipitates obtained by the addition of poly-L-seryl gelatin (●), poly-L-cysteinyl gelatin (○), gelatin (▲) and poly-L-tyrosyl gelatin A (Δ) to the antiserum to poly-L-seryl gelatin, as a function of the amount of precipitant.

Table 3. Composition of precipitates of the system poly-L-seryl gelatin-anti-poly-L-seryl gelatin

Precipitin reaction according to Maurer (1954).

Antigen added (μ g./ml. of serum)	Antigen precipitated (μ g.)	Antigen precipitated (%)	Antibody precipitated (μ g.)	Antibody/antigen in precipitate (w/w)
5	5.1	100	58	10.5
12	12.2	100	91	7.5
20	14.0	70	103	7.3
40	15.8	39.5	107	6.8
60	14.6	24.3	83.5	5.7
100	14.6	14.6	67.5	4.6
400	6.2	1.5	0	0

*Cross-precipitation and inhibition of the
gelatin-antigelatin reaction*

Antiserum to gelatin was obtained by immunization of three rabbits. The maximal amount of precipitate (118 μ g. of antibody/1 ml. of serum) was obtained by the technique of Maurer (1954) by adding 150 μ g. of the antigen to 4.5 ml. of the antiserum.

The results of precipitation of antibodies to gelatin with various polypeptidyl gelatins are given in Table 4. The extent of cross-precipitation decreases with the increase in the amount of amino acids attached to gelatin in the derivatives checked. Thus pTyrGelC, enriched with only 2.4% of tyrosine residues, is almost as good a precipitant as the homologous antigen, whereas pTyrGelA, enriched with 11.1% of tyrosine residues, causes a much smaller precipitation. The gelatin derivatives of L-tryptophan, L-lysine and L-glutamic acid, which cross-precipitated only to a limited extent, were enriched with more than 20% of the amino acid residues attached.

The following amino acid polymers and copolymers were tested for their possible inhibitory effect on the gelatin-antigelatin system: poly-L-proline; poly-L-hydroxyproline; random copolymers of L-proline and glycine; L-proline and sarcosine; L-proline, L-hydroxyproline and glycine; L-proline, L-glutamic acid and glycine; a low-molecular-weight polymer of the tripeptide ProGlyGly. None of the above substances had any influence on the homologous precipitin reaction.

DISCUSSION

The experiments described demonstrate that the enrichment of gelatin with an α -amino acid containing the cyclohexane ring in its side chain

converts it into a relatively powerful antigen. The amounts of antibodies precipitated compare favourably with those obtained by the precipitation of antibodies to polytyrosyl gelatins with the homologous antigens. It may be concluded that the previously observed (Sela & Arnon, 1960a) strong enhancement of the antigenicity of gelatin by attachment of aromatic amino acids is not due to their aromatic character. The common denominator for the two sets of results seems to be, rather, the increase in the rigidity of the antigen molecule. Although the cyclohexane ring is not as rigid as the aromatic benzene ring, it is known that certain energetically favoured configurations may become the stable ones. The chair conformation of cyclohexane is favoured energetically over that of a boat by approximately 6 kcal. For methylcyclohexane an equatorial conformation is more stable than the axial one by 1.8 kcal. (Beckett, Pitzer & Spitzer, 1947; Newman, 1956).

The attachment of serine to gelatin did not increase, in contrast to the attachment of cysteine, the extent of antigenicity. The serine derivative cannot form intramolecular bridges. On the other hand, a cysteine derivative readily forms such links. This is, again, in agreement with the suggested role of rigidity in the antigenicity of a molecule.

From the investigation of the cross-reaction of various polypeptidyl gelatins with the antiserum to gelatin it may be deduced that the attachment of peptide chains to gelatin interferes with the reactivity of the specific antigenic sites.

The use of synthetic polypeptides in inhibition experiments, intended to detect the chemical nature of the antigenically active sites of gelatin, was unsuccessful. As the precipitation of antigelatin with gelatin may be prevented by an adequate inhibitor, such as a chymotryptic digest

Table 4. *Cross-precipitation of antiserum to gelatin with various polypeptidyl gelatins*

Extinction of neutralized solutions of the precipitates obtained upon addition of the precipitants to 1 ml. of the antiserum.

Precipitant	Amount of precipitant added (μ g./ml. of serum)					
	7	11	27	33	67	110
Gelatin	0.089	0.142	0.181	0.178	0.171	0.087
pAlaGel	—	0.083	—	0.120	—	0.045
pCysGel	—	0.157	—	0.157	—	0.122
pGluGel	—	0.029	—	0.067	—	0.040
pLysGel	—	0.051	—	0.073	—	0.065
pPheGel	—	0.058	—	0.102	—	0.164
pProGel	—	0.130	—	0.142	—	0.070
pSerGelA	—	0.164	—	0.118	—	0.013
pTryGel*	—	0.052	—	0.070	—	0.123
pTyrGelA*	0.022	—	0.040	—	0.045	0.032
pTyrGelB*	0.087	—	0.154	—	0.148	—
pTyrGelC*	0.104	—	0.156	—	0.148	—

* Extinction is not corrected for the extinction of the precipitants.

of gelatin (Maurer, 1958), it may be concluded that the active antigenic site has a chemical structure much more intricate than that present in the various copolymers tested by us.

The sequence GlyProHypro is assumed to be of frequent occurrence in collagen and gelatin (Schroeder, Kay, LeGette, Honnen & Green, 1954; Rich & Crick, 1955). If the areas of rigidity responsible for the antigenicity of the gelatin molecule are due to its proline and hydroxyproline content, it is possible that a high-molecular-weight copolymer containing repeating sequences of GlyProHypro, when available, might inhibit the specific serological reaction.

SUMMARY

1. Poly-L-cyclohexylalanyl gelatins are relatively powerful antigens, as followed by the precipitin reaction. The antibodies to the two antigens investigated could be also cross-precipitated by other polycyclohexylalanyl derivatives.

2. The attachment of serine did not convert gelatin into a more powerful antigen. Antibodies to poly-L-seryl gelatin could not be detected by the usual precipitin techniques, but their presence could be shown by a method similar to that used by Maurer (1954) to demonstrate the antigenicity of gelatin.

3. In view of the increase in antigenicity as a result of the attachment of aromatic amino acids, cyclohexylalanine or cysteine to gelatin, it was concluded that areas of rigidity in the molecule are a necessary prerequisite for the enhancement of the antigenicity of gelatin.

4. The use of some synthetic polypeptides containing proline or hydroxyproline or both in inhibition experiments, intended to detect the chemical nature of the antigenically active sites of gelatin, was unsuccessful.

5. Various polypeptidyl gelatins were caused to cross-react with the antiserum to gelatin. An

increase in the amount of the amino acid attached to gelatin diminished considerably the extent of precipitation.

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