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Studies on the Chemical Basis of the Antigenicity of Proteins

1. ANTIGENICITY OF POLYPEPTIDYL GELATINS

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Most proteins have been tested for antigenicity, and practically all have been found to be antigenic, though to a varying extent. Although antigens of non-protein nature are also known, the proteins seem to be the most abundant and important antigenic substances yet studied. It is the purpose of this series of investigations to try to establish some correlation between the chemical nature of the protein antigens and their immunological activity.

To be antigenic, a molecule must possess a certain minimal degree of complexity and a certain mole-

cular size. On the other hand, it seems that only a part of the protein molecule is concerned with its antigenic specificity. The questions arise: what chemical structure must be present in the protein molecule to endow it with antigenic properties and what is the chemical nature of the moieties that can either enhance its antigenicity or influence its antigenic specificity?

These problems may be approached either by the immunological study of the products of partial degradation of antigen molecules or, as in the work reported here, by the investigation of the changes in the serological activity and specificity resulting from the attachment to the protein molecule of groups which may play a role in antigenicity.

Our detailed knowledge of haptenic specificity is due mainly to the pioneering work of Landsteiner (1945). In most studies the haptenic groups were derived from compounds not present normally in a protein, or, where they were composed of natural amino acids, the haptene was bound to the antigen through an azo bond (Landsteiner & van der Scheer, 1939). Serological investigations of substances prepared by attachment to proteins, through peptide bonds, of polypeptide chains composed of naturally occurring amino acids, would be of interest.

Polypeptidyl proteins may be obtained by causing proteins to react under mild conditions (aqueous solutions, neutral pH, low temperatures) with *N*-carboxy-α-amino acid anhydrides (Becker & Stahmann, 1953; Sela, 1954). In this way peptides of different sizes can be built on the free amino groups of the protein (Katchalski & Sela, 1958).

Immunological studies of polypeptidyl derivatives of strongly antigenic proteins, prepared by this method, have been reported recently. Thus the introduction of 171 and 210 glycyl units into bovine serum albumin produced a small but definite change in specificity (Makinodan, Becker, Wolfe & Stahmann, 1954). Stahmann, Tsuyuki, Weinke, Lapresle & Grabar (1955) investigated, by means of double diffusion in gels and by immunoelectrophoresis, the antigenic properties of polyleucyl, polyphenylalanyl, polyglutamyl and polylysyl derivatives of rabbit and bovine serum albumin. The immune sera prepared with polypeptidyl rabbit serum albumins cross-reacted with bovine serum albumin.

It has been considered whether the addition of polypeptides of different chemical composition to an immunologically rather inert protein carrier could enhance its low antigenicity and in this paper the first results of an immunological investigation of polypeptidyl derivatives of gelatin are reported.

The antigenicity of poly-α-amino acids has been investigated by several methods. Stahmann *et al.* (1955) reported that polyglutamic acid and polylysine are antigenic for the rabbit, though the antibodies could not be precipitated by the homologous antigens. On the other hand, Maurer (1957) did not observe detectable antibodies against polyglutamic acid in man, rabbit or guinea pig. Similarly, attempts to produce antibodies against various synthetic polymers of a single amino acid, by methods both *in vitro* and *in vivo*, were unsuccessful (Maurer, Subrahmanyam, Katchalski & Blout, 1959). Sela & Haurowitz (1958) failed to detect in rabbits antibodies to poly-*p*-arsanilazo-L-tyrosine by the usual precipitation techniques. Thus it seems that most poly-α-amino acids are not antigenic (Sela & Katchalski, 1959).

Gelatin is such a poor antigen that for many years its antigenicity escaped notice (Haurowitz, 1950; Boyd, 1956) and could be demonstrated only with refined precipitin techniques (Maurer, 1954*a*, *b*, 1955). A number of possible reasons for this low antigenicity may be postulated.

As a rigid structure seems to be essential for the antigenicity of proteins, it is quite possible that the lack of antigenicity of gelatin could be due to the lack of a definite structure in this heat-denatured protein, containing large amounts of glycine. The high percentage of proline and hydroxyproline could also be responsible for the lack of a configurational structure of the type encountered in other proteins (Szent-Gyorgyi & Cohen, 1957; Harrington & Sela, 1958). The deficiency of aromatic groups in gelatin has led many authors to postulate the necessity of such groups for antigenicity. As a matter of fact, the attachment of *O*-β-glucosido-*N*-benzyloxycarbonyl tyrosine (Clutton, Harington & Yuill, 1938) or *N*-benzyloxycarbonyltyrosine (Humphrey & Yuill, 1939; Micheel & Schallenberg, 1952) to gelatin gave substances which elicited the production of antibodies. These could not be precipitated with homologous antigens, but could be detected by precipitation with the respective derivatives of globulin. The benzyloxycarbonyl groups were not removed from the above antigens before the serological tests.

The possible enhancement of the antigenicity of gelatin by the attachment of tyrosine peptides was investigated by Sela, Katchalski & Olitzki (1956). Typical anaphylactic shocks were shown in guinea pigs sensitized with polytyrosyl gelatin (Sela, 1954), after intracardial injection of the same compound. On the other hand, no sensitization was observed by a similar treatment with gelatin alone or with a copolymer of tyrosine and aspartic acid. Poly-*p*-arsanilazotyrosyl gelatin acts as a strong antigen in rabbits, as shown by the precipitin test (Sela & Haurowitz, 1958).

The above preliminary investigations prompted us to undertake a systematic survey of the possible enhancement of the antigenicity of gelatin by the attachment of various polypeptidyl chains, which include derivatives of alanine, of the aromatic amino acids tyrosine, tryptophan and phenylalanine and of the strongly polar lysine and glutamic acid as well as of the sulphur-containing cysteine. The aromatic α-amino acids are present in gelatin only to a very small extent and cysteine was studied because gelatin contains almost no sulphur. The charged residues were added to test the common assumption that polar groups are of decisive significance for antigenic specificity. In order to see if the addition of any amino acid chain can enhance the antigenicity of gelatin, the relatively simple amino acid alanine was attached.

The amino acid with which gelatin was enriched was bound, in the form of short polypeptide chains, to as many as 75% of the amino groups of the original protein. Thus the polypeptides are linked to gelatin mainly through peptide bonds involving the ε-amino groups of lysine. Although such peptide bonds are not usually found in proteins, the existence of a peptide chain beginning at an ε-amino group of lysine in collagen has been reported recently (Mechanic & Levy, 1959).

This paper presents evidence that the polytyrosyl, polytryptophyl, polyphenylalanyl and, to some extent, polycysteinyl derivatives of gelatin behave as relatively strong antigens. The addition of polyglutamyl, polylysyl and polyalanyl chains do not significantly increase the antigenicity of gelatin.

EXPERIMENTAL

Materials

Amino acids and peptides are abbreviated as in Sanger (1952): Ala (alanine), Cys (cysteine), Glu (glutamic acid), Lys (lysine), Phe (phenylalanine), Ser (serine), Try (tryptophan) and Tyr (tyrosine). Gelatin (Gel), U.S.P. granular, was obtained from Fischer Scientific Co. Pittsburgh, Pa., U.S.A. After drying over phosphorus pentoxide *in vacuo*, it contained 18·3% of nitrogen (Eastoe, 1955, reported 18·11% for bone gelatin). Egg albumin, twice recrystallized, was prepared according to La Rosa (1927). Egg-white lysozyme, twice recrystallized, was obtained through the kindness of Dr W. F. Harrington. Edestin and the amino acids used in this study were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Poly-L-glutamic acid (number-average degree of polymerization 50) was synthesized according to Katchalski & Berger (1957). Multi-chain poly-L-glutamic acid (Yaron & Berger, 1958) of a molecular weight 170 000 was a gift from Mr A. Yaron. Tri-L-tyrosine ethyl ester (Neumann, Levin, Berger & Katchalski, 1959) was a gift from Dr Y. Levin. Glycyl-L-tyrosine was purchased from L. Light and Co. Ltd.

Polypeptidyl gelatins

All the polypeptidyl gelatins (Table 1) were prepared by the method of Becker & Stahmann (1953) as applied to gelatin by Sela (1954). Various N-carboxy-α-amino acid anhydrides in dioxan solution (1 g. of anhydride/20 ml. of anhydrous dioxan) were caused to react with gelatin in aqueous solution buffered at pH 7·0 with 0·05M-phosphate (1 g. of gelatin in 50 ml. of buffer). The reaction was carried out for 24 hr. at 2°, followed by an additional 24 hr. at room temperature.

Poly-DL-alanyl gelatin. This compound (pAlaGel) was prepared by adding 6 m-moles of N-carboxy-DL-alanine anhydride (Sela & Berger, 1955)/g. of gelatin. It was purified by dialysis against distilled water at 2° for 3 days. Under the polymerization conditions used, but in the absence of gelatin, N-carboxyalanine anhydride gives low-alanine peptides which passed through the dialysing membrane to an extent of 97% (Sela, Katchalski & Gehatia, 1956). The contents of the dialysis bag were lyophilized and stored at 2°. A chromatographic analysis of this material showed no traces of low-molecular-weight peptides. To determine the content of alanine, pAlaGel was hydrolysed with 6N-hydrochloric acid at 105° for 24 hr. The hydrolysate was subjected to paper electrophoresis on a Whatman no. 1 filter paper in 25 mM-phthalate buffer, pH 5·92 (5·10 g. of potassium hydrogen phthalate and 0·86 g. of sodium hydroxide in 1 l. of water), at a potential gradient of 10 v/cm., at 25°. After 2 hr. the paper was dried and chromatographed in the second dimension in butan-1-ol–acetic acid–water (50:12:50, v/v) for 48 hr. Alanine was estimated by ninhydrin colorimetry (Kay, Harris & Entenman, 1956) (see Table 1). To obtain information on the extent to which the ε-amino groups of gelatin served as initiators in the polymerization of N-carboxyalanine anhydride, pAlaGel was dinitrophenylated at pH 9·0 at 40° for 90 min. and hydrolysed. N-Dinitrophenyl-DL-alanine and ε-N-dinitrophenyl-L-lysine were determined colorimetrically (Levy, 1954; Anfinsen, Sela & Tritch, 1956). The ratio of dinitrophenyl (DNP)-alanine to ε-DNP-lysine is given in Table 1.

Poly-L-cysteinyl gelatin. This compound (pCysGel) was prepared by adding 5 m-moles of N-carboxy-L-cysteine anhydride (Berger *et al.* 1958)/g. of gelatin. The reaction mixture was solubilized by the addition of alkali to pH 11·0, followed by neutralization with hydrochloric acid. Poly-L-cysteine is not soluble in water in the neutral pH range (Berger, Noguchi & Katchalski, 1956). pCysGel was purified by dialysis against 0·02N-sodium thioglycollate for 3 days, followed by dialysis against water for 3 hr. The lyophilized material contained 2·2% of elementary sulphur (corresponding to 7·1% of cysteine residues). The content of sulphydryl groups was estimated spectrophotometrically by a modification (Sela, White & Anfinsen, 1959) of the *p*-chloromercuribenzoate method of Boyer (1954). It corresponded to 6·7% of cysteine residues. pCysGel was kept under vacuum at 2°; its sulphydryl content did not change for several months under these conditions.

Poly-L-glutamyl gelatin. This compound (pGluGel) was prepared by adding 4 m-moles of γ-benzyl-N-carboxy-L-glutamate anhydride (Katchalski & Berger, 1957)/g. of gelatin. After the completion of the reaction, 2 vol. of cold acetone was added and the precipitated polybenzylglutamyl gelatin was washed with acetone and dried *in vacuo* over phosphorus pentoxide. The benzyl groups

were removed with anhydrous hydrogen bromide in acetic acid at 2° for 65 hr. (Yaron & Berger, 1958). pGluGel was precipitated with ether, dissolved in water at pH 7·0 (by the cautious addition of N-sodium hydroxide), dialysed, lyophilized and stored as described for pAlaGel. The content of glutamic acid in pGluGel, as well as the ratio of DNP-glutamic acid to ε-DNP-lysine, were determined as for pAlaGel (see Table 1).

The electrophoretic mobility of pGluGel was compared with that of mixtures of gelatin and glutamic acid, and of gelatin and polyglutamic acid. The mixtures contained the same amount of glutamic acid as pGluGel. The measurements were carried out on 1% solutions, in a Kern LK 30 electrophoresis apparatus, in 0·05M-phthalate buffer, pH 6·0, and 0·05N-sodium chloride, at a potential gradient of 70v, at room temperature. pGluGel moved towards the anode as a single peak, and the mixtures of gelatin and glutamic acid or polyglutamic acid separated within 5 min.

Poly-L-*lysyl gelatin.* This compound (pLysGel) was prepared by adding 5 m-moles of ε-*N*-benzyloxycarbonyl-α-*N*-carboxy-L-lysine anhydride (Bergmann, Zervas & Ross, 1935)/g. of gelatin. After the completion of the reaction 2 vol. of cold acetone was added, and the precipitated polybenzyloxycarbonyl-lysyl gelatin was washed with acetone and dried *in vacuo* over phosphorus pentoxide. The benzyloxycarbonyl groups were removed with anhydrous hydrogen bromide (Ben-Ishai & Berger, 1952) at room temperature for 1 hr. pLysGel was precipitated with ether, dissolved in water at pH 7·0, dialysed, lyophilized and stored as described above. The content of lysine in pLysGel was determined similarly to that of alanine in pAlaGel, except that the chromatography in the second dimension was carried out in butan-1-ol–water–pyridine–acetic acid (30:29:20:6, by vol.).

The electrophoretic mobility of pLysGel was compared with that of a mixture of gelatin and polylysine hydrobromide (Katchalski, 1957) (number-average degree of polymerization 70) containing the same amount of lysine as pLysGel. The measurements were carried out on a Whatman no. 1 filter paper in 0·025M-phthalate buffer, pH 6·0, at a potential gradient of 10v/cm., at 25°. After 2 hr. the zones were detected with ninhydrin. pLysGel moved slowly as a round spot towards the cathode, and the mixture separated into a spot of gelatin that stayed at the origin and an elongated spot of polylysine that travelled quickly towards the cathode.

Poly-L-*phenylalanyl gelatin.* This compound (pPheGel) was prepared by adding 2 m-moles of *N*-carboxy-L-phenylalanine anhydride (Sela & Berger, 1955)/g. of gelatin. Poly-L-phenylalanine is insoluble in water (Katchalski & Sela, 1958). The products of the reaction of gelatin with higher amounts of *N*-carboxy-L-phenylalanine anhydride were similarly insoluble in water. pPheGel prepared as mentioned above could be dissolved by addition of alkali up to pH 11·0. No precipitate was formed upon neutralization. pPheGel was purified by dialysis, lyophilized and stored as usual. To determine the content of phenylalanine, pPheGel was hydrolysed with 6N-hydrochloric acid at 110° for 36 hr. The hydrolysate was chromatographed in butan-1-ol–acetic acid–water (50:12:50, by vol.) and valine, and phenylalanine plus leucine, were estimated by ninhydrin colorimetry (Kay *et al.* 1956). Since this solvent does not separate the last two amino acids, a similar analysis was performed on unmodified gelatin and the amount of attached phenylalanine was calculated by difference (see Table 1). The ratio of DNP-phenylalanine to ε-DNP-lysine was determined as for pAlaGel.

Poly-L-*tryptophyl gelatin.* This compound (pTryGel) was prepared by adding 5 m-moles of α-*N*-carboxy-L-tryptophan anhydride (Patchornik, Sela & Katchalski, 1954)/g. of gelatin. pTryGel obtained was solubilized by addition of alkali, followed by neutralization. It was purified by dialysis as usual and lyophilized. The content of tryptophan in pTryGel was calculated from the absorption of the material, as well as of its alkaline (barium hydroxide) hydrolysate, at 280 mμ (Beaven & Holiday, 1952).

Table 1. *Polypeptidyl gelatins*

A	B	C	D	E	F	G	H
Gelatin derivative (enriched with amino acid X)	Percentage of the X-amino acid residue in the original gelatin	Percentage of the X-amino acid residue in the gelatin derivative	Enrichment* $\left(\frac{C-B}{100-C}\right)\times 100$	Moles of amino acid attached/ 100 kg. of gelatin	Ratio of α-amino groups to ε-amino groups†	Number of moles of distinct polypeptide chains/ 100 kg. of gelatin‡ [30·6F/ (F+1)]	Average no. of amino acid residues/ peptide chain attached (E/G)
pAlaGel	9·0§; 8·7‖	18·4	11·8	166	3·65	24	6·9
pCysGel	0·0§	7·1	7·6	74	—	—	—
pGluGel	10·2§; 10·1‖	26·6	22·5	175	2·85	23	7·6
pLysGel	3·82§; 4·12‖	22·6	24·1	189	—	—	—
pPheGel	2·11§	8·85	7·4	50	2·45	22	2·3
pTryGel	0·06¶	25·4	34·0	183	—	—	—
pTyrGel	0·21§; 0·42‖	10·3	11·1	68	2·35	21	3·2

* Calculated assuming gelatin as 100%.
† Determined from the absorptions of the DNP-amino acid attached, and of ε-DNP-lysine, recovered from the hydrolysates of the dinitrophenylated gelatin derivatives.
‡ Calculated assuming 30·6 lysine residues/100 kg. of gelatin (Eastoe, 1955).
§ From Eastoe (1955).
‖ Determined by the authors under the same conditions as for the corresponding gelatin derivatives.
¶ From Goodwin & Morton (1946).

*Poly-*L*-tyrosyl gelatin.* This compound (pTyrGel) was prepared by adding 2·5 m-moles of *O*-benzyloxycarbonyl-*N*-carboxy-L-tyrosine anhydride (Katchalski & Sela, 1953)/g. of gelatin. After the completion of the reaction 2 vol. of cold acetone was added, and the precipitated poly-*O*-benzyloxycarbonyltyrosyl gelatin was washed with acetone and dried *in vacuo* over phosphorus pentoxide. The benzyloxycarbonyl groups were removed by the action of anhydrous hydrogen bromide in acetic acid (Sela, 1954) at room temperature for 1 hr. pTyrGel was precipitated with ether, dissolved in water at pH 7·0, dialyzed, lyophilized and stored in the cold as usual. Poly-L-tyrosine is not soluble in water in the neutral pH range. The content of tyrosine in pTyrGel was calculated from the absorption of an alkaline solution (pH 13·0) at 293·5 mμ. The ratio of DNP-tyrosine to ε-DNP-lysine was determined as described for pAlaGel (see Table 1).

*Poly-*DL*-seryl gelatin.* This compound (pSerGel) was prepared by adding 4 m-moles of *N*-carboxy-DL-serine amino acid anhydride (Berger *et al.* 1958)/g. of gelatin. The reaction product was dialysed and lyophilized. It contained 6·47% of serine residues, as determined by ninhydrin colorimetry after chromatography in pyridine–water (65:35, v/v) of the acid hydrolysate. This corresponds to an enrichment of 3·1%.

*Poly-*3-(p*-arsonylphenylazo)-*L*-tyrosyl gelatin.* This compound (pAzoTyrGel) was prepared similarly to other azo derivatives of polytyrosine (Sela & Katchalski, 1955). Diazotized *p*-arsanilic acid was coupled with a polytyrosyl gelatin containing 16% of tyrosine residues (1 mole of *p*-arsonylphenyl diazonium chloride/mole of tyrosine residue: thus pAzoTyrGel contains 31·4% of *p*-arsonylphenylazotyrosyl residues). pAzoTyrGel was precipitated with dilute hydrochloric acid, dissolved in dilute alkali, purified by dialysis against water and lyophilized. The material contained 5·8% of As (calc. for pAzoTyrGel containing 31·4% of *p*-arsonylphenylazotyrosyl residues of formula $C_{15}H_{13}O_5N_3As$: 6·0% As).

Labelled pTyrGel. This was prepared by iodination of pTyrGel with ^{131}I (50 μc/10 mg.) according to Talmage, Baker & Akeson (1954). The substance, isolated after exhaustive dialysis, had a radioactivity of 800 000 counts/min./mg.

Polypeptidyl egg albumins and edestins

These were prepared and analysed by techniques analogous to those of polypeptidyl gelatins. They are listed in Table 2.

Gelatin treated with anhydrous hydrogen bromide

Gelatin was dried at 60° *in vacuo* over phosphorus pentoxide. The dry sample was dissolved in acetic acid saturated with anhydrous hydrogen bromide. After 65 hr. it was precipitated with ether, dissolved in water at pH 7·0, dialysed as usual and lyophilized.

α-Amino acid copolymers

The *N*-carboxy-α-amino acid anhydride compounds of L-tyrosine (Berger *et al.* 1958), γ-benzyl-L-glutamate (Katchalski & Berger, 1957), L-leucine (Go & Tani, 1939), DL-alanine and L-phenylalanine (Sela & Berger, 1955) were used as starting monomers. They were all recrystallized repeatedly until no chlorine could be detected. In Table 3 are given the various copolymers prepared. The synthesis of tyrosine-containing copolymers was carried out at room

Table 2. *Polypeptidyl derivatives of other proteins*

A	B	C	D
Protein derivative (enriched with amino acid X)	Percentage of the X-amino acid residue in the original protein*	Percentage of the X-amino acid residue in the protein derivative	Enrichment† $\left(\frac{C-B}{100-C}\right)\times 100$
Poly-L-phenylalanyl egg albumin	6·82	14·1	8·5
Poly-L-tryptophyl egg albumin	1·10	17·6	20·0
Poly-L-tyrosyl egg albumin	3·32	14·8	13·5
Poly-L-phenylalanyl edestin	4·85	6·75	2·2
Poly-L-tyrosyl edestin	3·83	19·8	19·9

* From Tristram (1953). † Calculated assuming the original protein as 100%.

Table 3. *Copolymers of α-amino acids*

Copolymer of	Molar ratio of *N*-carboxy-α-amino acid anhydride molecules in the polymerization mixture	Molar ratio of the amino acid residues in the copolymer	Number-average degree of polymerization
L-Tyr, L-Glu	1:1	1:1·1	31
L-Tyr, L-Glu	1:3	1:4·0	31
L-Tyr, L-Glu	1:9	1:9·0	88
L-Tyr, DL-Ala	1:9	1:10·1	35
L-Phe, L-Glu	1:1	1:0·87	142
L-Phe, L-Glu	1:3	1:2·1	110
L-Phe, L-Glu	1:9	1:4·2	183
L-Tyr, L-Leu, L-Glu	1:1:4	1:1:1	39
L-Tyr, monoiodotyrosine, di-iodotyrosine, L-Glu	—	17:17:14:52	31

temperature in anhydrous dioxan (1 g. of N-carboxy-α-amino acid anhydride/20 ml. of dioxan), triethylamine being used as initiator. The benzylated copolymers were precipitated with water, dried *in vacuo* over phosphorus pentoxide and the benzyl groups were removed with anhydrous hydrogen bromide in acetic acid, as described for pGluGel. The copolymer of Tyr and Ala was precipitated from the polymerization mixture with ether, taken up in water, dialysed against water and lyophilized. The copolymers of phenylalanine and γ-benzylglutamate were prepared in anhydrous benzene and debenzylated by passing a stream of anhydrous hydrogen bromide through the reaction mixtures. The precipitation of the copolymers was completed with anhydrous ether; the copolymers obtained were washed with anhydrous ether and dried *in vacuo* over phosphorus pentoxide and potassium hydroxide.

The amino acid content of the various copolymers was determined by ninhydrin colorimetry (Kay *et al.* 1956) of the hydrolysates after chromatography in butan-1-ol–acetic acid–water (50:12:50, by vol.), or, for tyrosine-containing copolymers, by spectrophotometric analysis at pH 13·0 and 293·5 mμ. The number-average degrees of polymerization were obtained from amino nitrogen (Van Slyke) determinations.

Iodinated copolymer of L*-tyrosine and* L*-glutamic acid* (1:1·1)

This was synthesized similarly to polydi-iodotyrosine (Katchalski & Sela, 1953). The isolated iodinated copolymer was hydrolysed by reflux with saturated barium hydroxide solution. After neutralization with sulphuric acid and removal of the barium sulphate formed, the supernatant fluid was analysed chromatographically (butan-1-ol–acetic acid–water; 50:12:50, by vol.), and the amino acids, including monoiodotyrosine and di-iodotyrosine, were determined by ninhydrin colorimetry.

Methods

Immunization procedure. The antigens used were incorporated in a water in oil–adjuvant mixture, according to Salk & Laurent (1952). Ten parts of 5% gelatin-derivative solution, 1 part of Arlacel A (Atlas Powder Co., Wilmington, Del., U.S.A.) and 9 parts of Bayol F (Stanco Distributors, New York, U.S.A.) were homogenized by repeated filling and forcible ejection from a syringe. The adjuvant mixture contained 0·35 mg. of dead tubercle bacilli/ml.

Each material was injected into two rabbits. After pre-immunization bleedings, the antigen was administered into the thighs of the hind legs of the animals. Four injections of 0·5 ml. of the adjuvant mixture were given, at fortnightly intervals.

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. In preliminary tests qualitative precipitin reactions were carried out on sera of individual rabbits. In all experiments the antisera to a particular gelatin derivative behaved similarly. Thereafter such antisera were pooled before quantitative studies.

(*a*) In all cases increasing amounts of antigen dissolved in 0·75 ml. of aqueous 0·85% sodium chloride were added to test tubes containing 0·5 ml. of serum. In control experiments aqueous 0·85% sodium chloride solution was added in the absence of antigen. The contents of the tubes were mixed, placed in a water bath at 37° for 1 hr., and then in the cold room for 24 hr. or, in some cases, for 48 hr. The tubes were centrifuged at 2° and, whenever precipitates were formed, they were washed three times with chilled (2°) aqueous 0·85% sodium chloride, dissolved in 0·1N-sodium hydroxide (1·5 ml.) and neutralized with N-hydrochloric acid. The extinction of these solutions was read at 280 mμ. The amount of antigen in the precipitate was determined from the hydroxyproline content of the acid hydrolysate (6N-hydrochloric acid; 105°, 24 hr.). The antibody content was obtained from the extinction after deducting the calculated extinction of the antigen.

(*b*) Where no precipitates were formed under the above-described conditions, precipitin reactions were carried out essentially according to Maurer (1954*a*). After being stored in the cold room for about 10 days, each serum sample was centrifuged in the cold and the lipid was removed from the top. The serum was decanted and recentrifuged until perfectly clear. Increasing amounts of antigen dissolved in 0·5 ml. of aqueous 0·85% sodium chloride solution were added to test tubes containing 3 ml. of serum. The tubes were mixed, capped, placed in a water bath at 37° for 1 hr. and kept at 2° for 12 days, with mixing of the tubes daily. The tubes were centrifuged and the precipitates formed were treated and analysed as described under method (*a*). Precipitates formed in control test tubes were similarly analysed and the results deducted from the values obtained.

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 of the antigen–antibody precipitates, as well as in the hydrolysates of the gelatin derivatives themselves, by the method of Neuman & Logan (1950). Bone gelatin contains 13·3% of hydroxyproline (Eastoe, 1955).

Radioactivity. Radioactivity of antigen–antibody precipitates, as well as of the supernatant fluids, was measured in a well-type Tracerlab Scintillation Counter.

Spectrophotometric measurements. These were made on a Beckman model DU spectrophotometer, at approximately 25°, with quartz cells of 1 cm. light path. In the readings of solutions of antigen–antibody precipitates, cells with a capacity of 1 ml. were used.

RESULTS

Immunological response of polypeptidyl gelatins

All the polypeptidyl gelatins listed in Table 1 were tested for an immunological response. None of the materials gave any precipitate when added to pre-immunization sera. The antisera were subjected to the precipitin test. Positive reactions were obtained with polytyrosyl, polytryptophyl, polyphenylalanyl and polycysteinyl gelatins. The other gelatin derivatives, which gave no precipitations when examined by the usual methods, were tested by the more sensitive technique of Maurer (1954*a*).

Table 4. *Composition of precipitates of the system poly-L-tyrosyl gelatin–anti-poly-L-tyrosyl gelatin*

Antigen added (μg./ml. of serum)	Antigen precipitated, from hydroxyproline data* (μg.)	Antigen precipitated, from radioactivity data (μg.)	Antigen precipitated† (%)	Antibody precipitated‡ (μg.)	Antibody/antigen in precipitate (w/w)	Presence of antigen in the supernatant fluid	Presence of antibody in the supernatant fluid
50	52·0	49·6	100	210	4·2	−	+ +
100	73·7	56·2	65	268	4·85	−	+ +
250	95	92·5	37·5	369	3·94	−	+
500	95	97·0	19·2	481	5·02	±	−
750	83·4	81·4	11	481	5·84	+ +	−
1000	80	77·2	7·9	449	5·68	+ + +	−
1500	64·4	54·4	4	372	6·19	.	.

* Bone gelatin contains 13·3% of hydroxyproline (Eastoe, 1955).
† Calculated from the average of data obtained by the hydroxyproline and the radioactivity methods.
‡ Average of results of three experiments.

Polytyrosyl gelatin. Table 4 gives the analysis of the precipitates from the antiserum against polytyrosyl gelatin tested with the homologous antigen. In this case the amount of antigen in the precipitate was determined not only from its hydroxyproline content, but also by using labelled antigen. Because of the presence of tyrosine in pTyrGel it was possible to iodinate it with ^{131}I. The results obtained with both methods are in reasonable agreement. The amount of antibody was derived from the extinction of solutions of the precipitates at 280 mμ, after deducting the extinction of the antigen present. The precipitin curve is given in Fig. 1.

The antibodies to pTyrGel are very specific. Anti-pTyrGel was precipitated only by its homologous antigen, by other tyrosine derivatives of gelatin containing differing amounts of tyrosine (see the next paper, Arnon & Sela, 1960) and by pAzoTyrGel. No precipitation occurred when any of the following materials was added, at three different concentrations (20, 200 and 1000 μg.), to 0·2 ml. of the antiserum: gelatin, pTryGel, pPheGel, pGluGel, pLysGel, pAlaGel, egg albumin, poly-L-tyrosyl egg albumin, tyrosine, glycyl-L-tyrosine, tri-L-tyrosine as well as all of the copolymers containing tyrosine or di-iodotyrosine mentioned in Table 3.

All those materials that did not precipitate anti-pTyrGel were tested for their ability to inhibit the specific precipitin reaction. Table 5 summarizes the inhibition experiments for copolymers of amino acids, as well as poly-L-tyrosyl egg albumin and poly-L-tyrosyl edestin. As seen from Table 5, the inhibition was successful only for copolymers rich in tyrosine or polytyrosyl proteins. No inhibition was observed when tyrosine, trityrosine, gelatin, various polypeptidyl gelatins or polyglutamic acid was added.

Polytryptophyl gelatin. Table 6 gives the analysis of the precipitates from the antiserum against

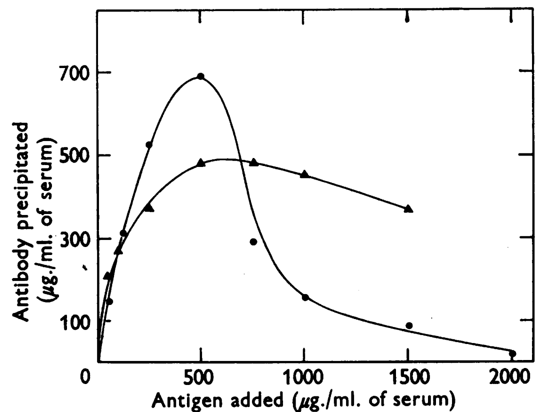

Fig. 1. Homologous precipitin curves of poly-L-tryptophyl gelatin (●) and poly-L-tyrosyl gelatin (▲).

Table 5. *Inhibition of poly-L-tyrosyl gelatin–anti-poly-L-tyrosyl gelatin precipitation*

Inhibitor	Amount added (μg./0·2 ml. of serum)	Precipitate
Poly-L-tyrosyl egg albumin	200	±
	1000	−
Poly-L-tyrosyl edestin	200	±
Copolymer Tyr.Glu (1:1)	50	+ + +
	200	±
	1000	−
Copolymer Tyr.Glu (1:4)	100	+ + +
	300	+ +
	2000	+
Copolymer Tyr.Glu (1:9)	150	+ + +
	500	+ + +
	3000	+ + +
Copolymer Tyr.Leu.Glu (1:1:1)	100	+ +
	250	+
	1000	±
Copolymer Tyr.Ala (1:10)	250	+ + +
	1000	+ + +
Iodinated Tyr.Glu copolymer	100	+ + +
	300	+
	1000	−

polytryptophyl gelatin, tested with the homologous antigen. The precipitin curve is given in Fig. 1. The antibodies to pTryGel are specific, similarly to the antibodies to pTyrGel. In addition to the homologous antigen, they could be precipitated only by poly-L-tryptophyl egg albumin. Fig. 2 gives the extinction of the solutions of the precipitates of poly-L-tryptophyl egg albumin–anti-pTryGel. A similar curve for pTryGel–anti-pTryGel is given for comparison.

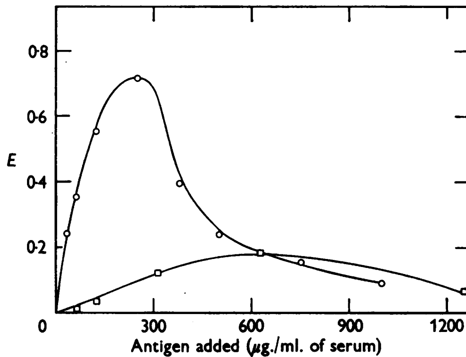

Fig. 2. Extinction of neutral solutions of precipitates. obtained by the addition of poly-L-tryptophyl gelatin (○) and poly-L-tryptophyl albumin (□) to the antiserum to poly-L-tryptophyl gelatin, as a function of the amount of the precipitant.

Polyphenylalanyl gelatin. The analysis of the precipitates from the antiserum against polyphenylalanyl gelatin tested with the homologous antigen is given in Table 6. In Fig. 3 is shown its precipitin curve. No precipitation of antibodies to pPheGel was observed when gelatin, egg albumin, pTyrGel, pGluGel, poly-L-phenylalanyl egg albumin, as well as all the copolymers containing phenylalanine mentioned in Table 3, were added. All these materials were tested subsequently for their ability to inhibit the precipitin reaction. As seen from Table 7, the only material that caused complete inhibition was a copolymer of L-phenylalanine and L-glutamic acid, in a residue molar ratio 1:0·87.

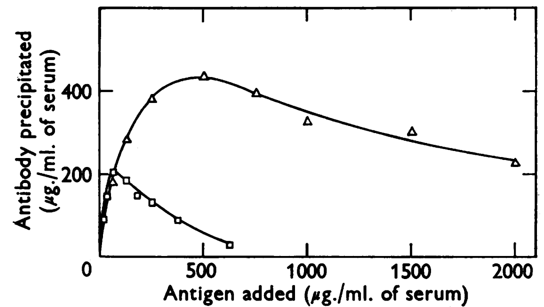

Fig. 3. Homologous precipitin curves of poly-L-phenylalanyl gelatin (△) and poly-L-cysteinyl gelatin (○).

Table 6. *Composition of precipitates of the systems pTryGel–anti-pTryGel, pPheGel–anti-pPheGel and pCysGel–anti-pCysGel*

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated (μg.)	Antigen precipitated (%)	Antibody precipitated (μg.)	Antibody/antigen in precipitate (w/w)
pTryGel	60	45·6	76	144	3·16
	125	49·8	40	308	6·2
	250	70·4	27	522	7·4
	500	91·2	18·5	668	7·3
	750	66·4	8·9	288	4·35
	1000	45·6	4·6	153	3·4
	1500	29·0	1·9	89	3·1
	2000	16·6	0·8	14·6	—
pPheGel	60	53	88·3	182	3·4
	125	72	57·5	284	3·9
	250	103	41·2	380	3·7
	500	127	25·4	434	3·5
	750	154	20·5	396	2·6
	1000	147	14·7	326	2·25
	1500	137	9·1	304	2·2
	2000	127	6·3	227	2·1
pCysGel	12·5	12·9	100	90	6·9
	31	30·5	98	147	4·8
	62·5	43·4	69·5	206	4·7
	125	39·7	31·7	183	4·6
	187·5	35·4	18·9	148	4·2
	250	31·8	12·7	132	4·15
	375	22·0	5·9	90	4·1
	625	4	0·6	29	—

Polycysteinyl gelatin. The composition of the precipitates of the pCysGel–anti-pCysGel system is given in Table 6. The precipitin curve is shown in Fig. 3. The form of the curve is similar to those obtained for pTyrGel, pTryGel and pPheGel, but the amount of the precipitated antibody is considerably smaller. No precipitation of antibodies to pCysGel was observed when gelatin, pTyrGel, pSerGel or egg albumin was added. When these materials were tested, only pSerGel, at 1000 μg., caused complete inhibition, whereas gelatin and pTyrGel, at 1000 μg., only partially inhibited the pCysGel–anti-pCysGel precipitation.

Other polypeptidyl gelatins. Antisera against pAlaGel, pGluGel and pLysGel, as well as against gelatin treated with anhydrous hydrogen bromide, gave no precipitates with the homologous antigens when subjected to the usual tests. Nor was any precipitation observed when the antisera were brought into cross-reaction with the various gelatin derivatives. The precipitin test was therefore performed in a way similar to that used by Maurer (1954*a*) to demonstrate the antigenicity of unmodified gelatin. Table 8 gives the amount of antibodies that could be precipitated by the homologous antigens in the equivalence zone. Maurer (1954*b*) found 78 μg. of antibody against unmodified gelatin in 1 ml. of rabbit antiserum. The data of Table 8 therefore indicate that the treatment with anhydrous hydrogen bromide did not alter the extent of antigenicity of gelatin. In view of this it may be concluded that any changes observed in the antigenicity of pTyrGel, pGluGel and pLysGel, as compared with gelatin, are due to the enrichment of gelatin with the respective amino acids and not to the treatment with anhydrous hydrogen bromide during their preparation.

The antibodies to polyglutamyl gelatin were also precipitated by gelatin, by multi-chain polyglutamic acid and by a gelatin derivative enriched both with tyrosine and glutamic acid (pGluTyrGel, see the next paper, Arnon & Sela, 1960). Precipitation curves for all the above are given in Fig. 4.

Table 7. *Inhibition of pPheGel–anti-pPheGel precipitation*

Inhibitor	Amount added (μg./0·2 ml. of serum)	Precipitate
Gelatin	50	+ + +
	200	+ +
	1000	+
Egg albumin	50	+ + +
	200	+ + +
	1000	+ + +
Poly-L-phenylalanyl egg albumin	50	+ + +
	200	+ +
	1000	+
pTyrGel	50	+ + +
	200	+ +
	1000	+
pGluGel	50	+ + +
	200	+ +
	1000	+
Copolymer Phe.Glu (1:1)	50	+ +
	200	+
	1000	–
Copolymer Phe.Glu.(1:2)	50	+ + +
	200	+ +
	1000	+
Copolymer Phe.Glu (1:4)	200	+ + +
	500	+ + +
	2000	+ + +

Non-precipitable antibodies

As seen from Tables 4, 6 and 8, the precipitates in the equivalence zone contain only 20–70 % of the total antigen added. In this region no precipitate occurs upon addition of either antigen or antibody to the supernatant fluid. This implies heterogeneity of antigen or of antibody. Gelatin is not a monodisperse substance, and its use as a multi-functional initiator in the polymerization of *N*-carboxy-α-amino acid anhydrides contributes, though in a limited way (Katchalski, Gehatia & Sela, 1955), to the polydispersity of the product. Nevertheless, the fact that at high antibody–antigen ratios all the antigen is found in the precipitate suggests that the antibodies to the various polypeptidyl gelatins are at least of two types, namely precipitating and non-precipitable.

To explore the possibility that the non-precipitable antibody in the antiserum against pTyrGel might be an antibody to unmodified gelatin, the following experiment was performed: A great excess of gelatin was added to antiserum against

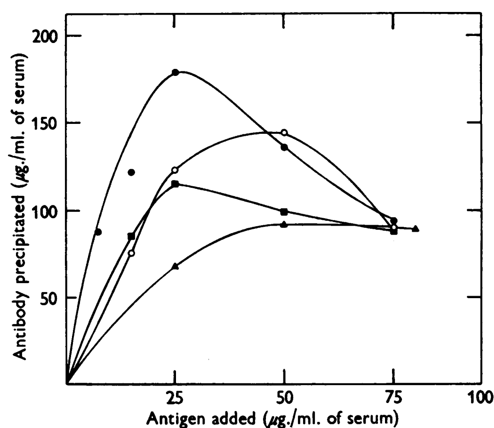

Fig. 4. Precipitin curves of poly-L-glutamyl gelatin (●), gelatin (■), multichain poly-L-glutamic acid (▲) and copoly-(L-glutamyl, L-tyrosyl)gelatin (○) with the antiserum to poly-L-glutamyl gelatin.

Table 8. *Antibodies precipitated by gelatin treated with hydrogen bromide, pAlaGel, pGluGel and pLysGel from the respective antisera*

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated (μg.)	Antigen precipitated (%)	Antibody precipitated (μg.)	Antibody/ antigen in precipitate (w/w)
Gelatin–HBr	15	—	—	32	—
	30	8	28	93	11·6
	50	—	—	57	—
pAlaGel	15	—	—	85	—
	30	20	67	192	9·6
	50	—	—	152	—
pGluGel	15	—	—	93	—
	30	19	63	183	9·6
	50	—	—	130	—
pLysGel	15	13	86	133	10·2
	30	—	—	174	—
	50	27	54	179	6·7

Table 9. *Amount of antibodies precipitated from* 1 *ml. of antiserum in the equivalence zone*

	Precipitate obtained by the usual technique		Precipitate obtained by Maurer's technique	
Antigen	Antigen added to cause maximal precipitation (μg.)	Antibody precipitated in the equivalence zone (μg.)	Antigen added to cause maximal precipitation (μg.)	Antibody precipitated in the equivalence zone (μg.)
pTyrGel	500	481	500	570
pTryGel	500	668	—	—
pPheGel	500	434	—	—
pCysGel	65	206	—	—
pLysGel	—	0	50	179
pGluGel	—	0	30	183
pAlaGel	—	0	30	192
Gelatin–HBr	—	0	30	93

pTyrGel. After incubation for 1 hr., an amount of pTyrGel that should give the maximal precipitation was added. The precipitate, composed of pTyrGel and precipitating antibody against pTyrGel, was removed, and the supernatant fluid was tested for the presence of free pTyrGel by addition of more antiserum. No precipitate was formed.

If the non-precipitable antibodies had been antibodies to gelatin, they would have been bound strongly by the gelatin added at the beginning of the experiment and would not have been available for pTyrGel. The fact that no free pTyrGel could be detected in the supernatant fluid suggests that the antiserum to pTyrGel indeed contains non-precipitable antibodies which are specific to pTyrGel and not to gelatin.

A parallel experiment with the pPheGel system yielded similar results. A more direct proof of the existence of a soluble antigen–antibody complex in the supernatant fluid at the equivalence zone, based on electrophoretic experiments with a radioactively labelled antigen, will be given in the next paper (Arnon & Sela, 1960).

Comparison of the various polypeptidyl gelatins

The results obtained with the various polypeptidyl derivatives of gelatin are collected in Table 9. With a good antigen, e.g. pTyrGel, the use of Maurer's technique increases only to a small extent the amount of antibody precipitated from the serum. On the other hand, poor antigens, which do not cause any precipitation under the usual conditions, are able in Maurer's technique to precipitate amounts of antibodies similar to those obtained, e.g. with pCysGel, by the usual techniques. Thus it seems that the criteria for the antigenicity of a protein should include not only the amount of antibodies that can be elicited by the antigen, but also the conditions under which these antibodies can be detected.

DISCUSSION

The experiments described demonstrate that enrichment of gelatin with α-amino acids containing aromatic structures in their side chain converts gelatin into relatively powerful antigens. It cannot

be decided yet if this enhancement results specifically from the aromatic character of the amino acids attached or if it is an example of a more general enhancement in antigenicity due to an increase in the rigidity of the molecule. Studies now in progress on the antigenicity of poly*cyclo*hexylalanyl gelatin will enable us to answer this question.

The attachment of cysteine residues to gelatin also contributed to an increase in antigenicity although to a much smaller degree. This, again, might be due to an increase in the rigidity of the protein. Even though the content of sulphydryl groups in pCysGel 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*.

The very limited change in the antigenic potency of gelatin caused by enrichment with lysine or glutamic acid suggests that the polar amino acid residues have no significant enhancing ability although they may still exercise considerable influence on the specificity of antigens.

The antibodies to all the polypeptidyl gelatins possess a very narrow specificity, being precipitated almost exclusively by the homologous antigens. Only with anti-pTryGel was there cross-reaction with poly-L-tryptophyl egg albumin. It should be noticed that the enrichment with tryptophan was higher than in any other gelatin derivative.

In all cases we found specific inhibitors that could interfere with the precipitation of the antibodies by the homologous antigens. From the chemical structure of these inhibitors, information can be obtained about the size and character of the reacting site on the antibody molecule, as well as on the chemical nature of the immunologically active site on the antigen molecule. The pTyrGel and pPheGel systems could be inhibited by copolymers rich in tyrosine and phenylalanine respectively. Such copolymers possess a random sequence, i.e. they contain in their chains regions of repeating units of tyrosine or of phenylalanine. As copolymers with a lower ratio of the aromatic α-amino acid, where the chance of finding adjacent residues of tyrosine or phenylalanine in the chain is very small, have no inhibiting properties, it must be assumed that sequences of several tyrosine or phenylalanine residues are necessary for the inhibition.

The inhibition of a precipitin reaction involving one gelatin derivative, by various other modified gelatins, shows that gelatin contributes towards the specificity of the immunological reaction. Thus anti-pPheGel is inhibited not only by the pTyrGel (enriched with aromatic groups) but also by pGluGel and by gelatin itself. Moreover, with Maurer's precipitin technique, gelatin precipitated anti-pGluGel.

All the gelatin derivatives investigated gave rise to non-precipitable as well as precipitating antibodies. The presence of non-precipitable antibodies in immunological systems has been repeatedly reported. The techniques used were based mainly on the labelling of the antigen with ^{131}I (Burtin, 1955; Feinberg, 1958). However, it is conceivable that the persistence of radioactivity in supernatant fluids was due to inhomogeneous labelling, so that one small fraction of antigen is so highly labelled that it reacts as a heterologous cross-reacting antigen. An enzyme which is not completely inhibited by antibody permits the detection of such soluble complexes without any chemical modification of the antigen (Cinader, 1957).

The amount of the antigen in the precipitate and in the supernatant fluid was here calculated from the hydroxyproline content. This method has been used previously by Maurer (1954*a*), who showed, while studying the antigenicity of gelatin in man, that in the equivalence zone only 50% of the gelatin was present in the precipitate. The reasonably good agreement between the data obtained for pTyrGel with both the hydroxyproline and the radioactive-iodine techniques suggests that the danger of uneven isotopic labelling is small in these systems.

It was shown in this study that the attachment of some non-antigenic peptides to a poorly antigenic protein yields potent antigens. From various inhibition experiments it appears that gelatin is definitely involved in the immunologically active sites of these antigens. On the other hand, the peptide chains attached contribute strongly to the immune specificity. The role of the peptides and of gelatin in the specificity of polypeptidyl gelatins is discussed in detail in the next paper (Arnon & Sela, 1960).

SUMMARY

1. Several polypeptidyl derivatives of gelatin and albumin have been synthesized and characterized. They include polyalanyl, polyglutamyl, polylysyl, polycysteinyl, polytyrosyl, polytryptophyl and polyphenylalanyl gelatins. Various copolymers of α-amino acids have also been prepared and analysed.

2. The enrichment with tyrosine, tryptophan and phenylalanine converted gelatin into relatively powerful antigens, as followed by the precipitin reaction. The attachment of cysteine to gelatin caused a limited enhancement of antigenicity.

3. Antibodies to polyalanyl, polyglutamyl and polylysyl gelatins 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*a*) to demonstrate the antigenicity of gelatin.

4. The antibodies to polypeptidyl gelatins could be precipitated almost exclusively by the homologous antigens. However, it was possible to interfere with the precipitin reactions by means of specific inhibitors. From the inhibition experiments it was concluded that the polypeptidyl chains, and in some cases also gelatin itself, contribute towards the specificity of the antigens.

5. The presence of non-precipitable as well as precipitating antibodies to all the gelatin derivatives investigated was shown.

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