3. The ratios of the amidase, amide-transferase and acid-transferase activities exhibited by each enzyme varied in an identical manner with different substrates. Both enzymes exhibited identical kinetic properties.

4. The amidase activity of both enzymes was non-competitively inhibited by urea (K_i 1·1 mM) and by *N*-methylurea; these inhibitions were reversed by hydroxylamine. Thiourea did not inhibit.

5. The amide-transferase and acid-transferase activities of both enzymes were inhibited by iodoacetate and by *p*-hydroxymercuribenzoate: the inhibition caused by the latter compound was reversed by cysteine. Both activities were also inhibited by low concentrations of fluoride in the presence but not in the absence of hydroxylamine.

6. These results support the view that the enzyme from cells grown on acetamide was identical with that from cells grown on acetate. The properties of the enzyme were those of an acyltransferase, in which acyl-enzyme complexes were split by hydroxylamine or water.

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Antigenicity of Some New Synthetic Polypeptides and Polypeptidyl Gelatins

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Synthetic polypeptides may be immunogenic and such synthetic immunogens possess well-defined antigenic specificities (Sela, Fuchs & Arnon, 1962; Fuchs & Sela, 1963; Gill & Doty, 1961; Gill & Matthews, 1963; Maurer, 1963; Maurer, Gerulat & Pinchuck, 1962). The availability of synthetic antigens allows systematic variation of different structural parameters of the molecule so that these can be correlated with antigenic properties (Sela, 1962). For this purpose it is desirable to deal with as simple a system as possible that would possess well-defined immunogenic regions. In two previous papers (Sela *et al.* 1962; Fuchs & Sela, 1963) we discussed the immunogenicity and antigenic specificity of various linear and multichain synthetic polypeptides in which tyrosine was the immunogenic factor. The copolypeptides investigated contained all or some of the amino acids tyrosine, glutamic acid, alanine and lysine.

Conclusions about the role of shape and size in the antigenic features of a molecule, as well as of the accessibility of the antigenically important area of the molecule to the site of the biosynthesis of the antibody, were presented by Sela *et al.* (1962). Tyrosine was present in all of the polymers found to be immunogenic. Thus, multichain poly-DL-alanine (pAla--pLys*) is not immuno-

* For nomenclature, see Sela *et al.* (1962).

genic, nor does it become immunogenic upon attachment of peptides of glutamic acid. In contradistinction, the attachment of either tyrosine peptides or peptides of tyrosine and glutamic acid converted multichain poly-DL-alanine into immunogenic substances.

Tyrosine was not expected to be unique in endowing a molecule with immunogenic properties, as earlier studies (Sela & Arnon, 1960*a*) have shown that enrichment of gelatin with tyrosine, tryptophan or phenylalanine enhanced significantly the antigenicity of gelatin. The attachment of the non-aromatic cysteine and cyclohexylalanine (Sela & Arnon, 1960*b*) peptides to gelatin also increased the immune response.

In this paper we report the contribution of different amino acids present in the immunogenically important areas of the molecule to the antigenic response. The attachment of peptides of methionine, of leucine and glutamic acid, and of lysine and glutamic acid to gelatin resulted in enhancement of antigenicity, whereas the attachment of copolypeptides of glutamic acid and phenylalanine, histidine, leucine or lysine to the synthetic multichain poly-DL-alanine converted it into immunogens. Some linear synthetic polypeptide antigens devoid of tyrosine were described by Gill & Doty (1961), Gill & Matthews (1963) and Maurer (1962, 1963).

MATERIALS

Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex. Five-times-recrystallized ribonuclease was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. The samples of gelatin, poly-L-tyrosyl gelatin (pTyrGel), egg albumin, linear poly-L-glutamic acid (number-average degree of polymerization, *n* 50) and copolymers of L-phenylalanine and L-glutamic acid [p(Phe,Glu)] in residue molar ratios 1:2·1 (*n* 110) and 1:4·2 (*n* 183) were described by Sela & Arnon (1960*a*). A copolymer of L-leucine and L-glutamic acid in a residue molar ratio 1:0·8 (*n* 190) [p(Leu,Glu)] was described by Sela & Steiner (1963). 1-Benzyl-*N*-carboxy-L-histidine anhydride hydrochloride, prepared according to Patchornik, Berger & Katchalski (1957), was a gift from Dr S. Shaltiel. Samples of poly-L-phenylalanine, poly-L-lysine hydrobromide and a copolymer of L-lysine and L-glutamic acid in a residue molar ratio 1:1 [p(Lys,Glu)] were obtained from the collection of the Department of Biophysics. The synthesis and characterization of the samples 34, pGlu-pAla--pLys, and 35, p(Tyr,Glu)-pAla--pLys, were given by Sela *et al.* (1962).

Several multichain copolymers, listed in Table 1, were prepared by allowing multichain poly-DL-alanine to react with various *N*-carboxy-α-amino acid anhydrides. These included the *N*-carboxy anhydrides of L-phenylalanine (Sela & Berger, 1955), L-leucine (Farthing, 1950), L-methionine (McDonald, 1954), 1-benzyl-L-histidine (Patchornik *et al.* 1957), γ-benzyl-L-glutamate (Katchalski & Berger, 1957) and N^{ϵ}-benzyloxycarbonyl-L-lysine (Katchalski & Sela, 1958). The multichain copolymers were prepared and analysed as described by Sela *et al.* (1962).

To obtain the unprotected histidine-containing copolymer [220, p(His,Glu)-pAla--pLys], the protecting γ-benzyl groups were first removed from the γ-benzyl-L-glutamate residues by treatment with anhydrous hydrogen bromide in acetic acid for 72 hr. at 2° (Yaron & Berger, 1958); the residual benzyl groups were then removed from the imidazole nitrogen of histidine by treatment with sodium in liquid ammonia (Patchornik *et al.* 1957). The unprotected copolymer was dissolved in 1N-hydrochloric acid, dialysed against several changes of distilled water and the content of the dialysis bag was freeze-dried.

Most multichain polypeptide antigens described in this paper were prepared from 208, pAla--pLys. This particular sample of multichain poly-DL-alanine had an average mol. wt. 27800, calculated from an intrinsic sedimentation coefficient $S_{20,w}$ 1·92S, an intrinsic diffusion coefficient $D_{20,w}$ $6{\cdot}0 \times 10^{-7}$ cm.²/sec., and a partial specific volume 0·72. This volume was computed from the partial specific volumes of the component amino acid residues and their proportion by weight in the polymer (Sela *et al.* 1962). The sample 107, p(Leu,Glu)-pAla--pLys, had an average mol. wt. 31600, calculated from an intrinsic sedimentation coefficient $S_{20,w}$ 2·6S, an intrinsic diffusion coefficient $D_{20,w}$ $6{\cdot}9 \times 10^{-7}$ cm.²/sec. and a computed partial specific volume 0·71. The partial specific volume of the leucine residue was taken as 0·90 (Cohn & Edsall, 1943).

Polypeptidyl gelatins were prepared by the method of Becker & Stahmann (1953) and Sela (1954). They were analysed according to the methods described by Sela & Arnon (1960*a*) and are listed in Table 2.

METHODS

Immunization procedure. The antigens used were administered in complete Freund adjuvant by intramuscular injections into the thighs of the hind legs of rabbits. Each material tested was injected into four rabbits. The complete schedule of immunization was that described by Sela *et al.* (1962). If a negative response still persisted after four intramuscular injections, the animals were injected intravenously for 4 consecutive days with 0·5 ml. of a 0·2% antigen solution in aqueous 0·9% sodium chloride.

The animals were bled weekly, starting 10 days after the third injection.

Precipitin studies. Homologous and heterologous precipitin reactions were carried out as described by Fuchs & Sela (1963). The amount of antibody present in each precipitate was determined by using $E^{1\%}_{1\,cm.}$ 14·0 for 280 mμ (Porter, 1957). The antigens used in this study do not contribute significantly to *E* at this wavelength.

Inhibition studies. These were also carried out as described by Fuchs & Sela (1963). When poly-L-phenylalanine, which is not water-soluble, was examined for its inhibitory effect on the homologous system of a phenylalanine-containing polymer, the inhibition test was carried out in the following way: increasing amounts of the polymer were incubated with the antiserum; the mixture was stirred during the incubation; after centrifugation the optimum amount of the homologous antigen was added to the supernatant.

Haemagglutination. Haemagglutination was carried out according to a modification (Stavitsky, 1954) of the method of Boyden (1951). The antigen was adsorbed, at pH 7·2, on the surface of sheep red blood cells treated with tannic acid. Sodium chloride solution buffered at pH 7·2 was prepared according to Stavitsky (1954). The cells thus sensitized are agglutinated by diluted specific antisera. The titre of the antisera tested by this technique is expressed by the reciprocal of the highest dilution at which agglutination occurred.

Spectrophotometric measurements. These were made on a Beckman model DU spectrophotometer, at approx. 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.

Sedimentation and diffusion measurements. These were performed in a Spinco model E ultracentrifuge as described by Sela *et al.* (1962).

RESULTS

The immunogenic capacity of the various amino acids investigated was tested after their attachment, in peptide form, to carriers of high molecular weight. We chose for this purpose either the slightly antigenic gelatin (Maurer, 1954) or the non-antigenic multichain poly-DL-alanine (pAla--pLys; Sela *et al.* 1962).

The polymers and the peptidyl gelatins tested for immunogenicity in rabbits are listed in Tables 1 and 2. None of the materials gave any precipitate when added to pre-immunization sera, nor did the antisera obtained cross-precipitate with the native protein antigens: egg albumin, bovine serum albumin and pancreatic ribonuclease.

Synthetic polypeptide containing phenylalanine. The attachment of phenylalanine peptides to gelatin resulted in an enhanced antigenic capacity of the product as compared with unmodified gelatin (Sela & Arnon, 1960*a*). It was therefore expected that the attachment of phenylalanine to the non-antigenic multichain polypeptide pAla--pLys would result in its conversion into an immunogen. To endow the phenylalanine-containing synthetic substance with good solubility properties [based on the knowledge that pGlu-pAla--pLys is not antigenic in rabbits, whereas p(Tyr,Glu)-pAla--pLys is a powerful antigen of defined specificity], we have prepared a polymer in which peptides containing both L-phenylalanine and L-glutamic acid are attached to the non-antigenic carrier, and we have investigated its immunological properties. The resulting macromolecule, denoted 223, p(Phe,Glu)-pAla--pLys, and containing 14·8 % of phenylalanine residues, is immunogenic in rabbits as shown in Fig. 1.

The antigenic specificity of p(Phe,Glu)-pAla--pLys is also illustrated in Fig. 1. The antibodies cross-react best with p(Tyr,Glu)-pAla--pLys, somewhat less with pGlu-pAla--pLys and least with

Table 1. *Preparation and amino acid content of multichain copolymers*

No. and designation of sample	Multifunctional initiator: No. and designation	Multifunctional initiator: Wt. (g.) and vol. (ml.) of buffer	Amino acid X*	Wt. (g.) of *N*-carboxy anhydrides of: X	γ-Benzyl-L-Glu	Molar ratio of amino acid residues in the copolymer: L-Lys	X†	L-Glu	DL-Ala	Wt. (%) of the X amino acid residues in the copolymer
104, pAla--pLys	pLys	1·25; 500	DL-Alanine	15	—	1	—	—	14·0	—
208, pAla--pLys	pLys	2·2; 600	DL-Alanine	29	—	1	—	—	19·4	—
107, p(Leu,Glu)-pAla--pLys	104, pAla--pLys	1·25; 130	L-Leucine	0·6	1·0	1	2·04	2·78	12·7	14·2
203, p(Lys,Glu)-pAla--pLys	208, pAla--pLys	2; 130	L-Lysine	1·25‡	1·6	1§	—	1·05	5·35	19·9§
204, pMet-pAla--pLys	208, pAla--pLys	1·25; 80	L-Methionine	0·4	—	1	1·30	—	17·8	10·8
220, p(His,Glu)-pAla--pLys	208, pAla--pLys	2; 180	L-Histidine	1·1‖	1·4	1	1·10	3·7	18·8	7·2
223, p(Phe,Glu)-pAla--pLys	208, pAla--pLys	2·7; 250	L-Phenylalanine	0·9	1·8	1	2·40	4·0	19·6	14·8

* The amino acid reacted in the form *N*-carboxy anhydride with the multifunctional initiator (excluding glutamic acid).
† Other than lysine or alanine.
‡ N^{ε}-Benzyloxycarbonyl-N^{α}-carboxy-L-lysine anhydride.
§ Total amount of lysine in the molecule.
‖ 1-Benzyl-N^{α}-carboxy-L-histidine anhydride hydrochloride.

Table 2. *Polypeptidyl gelatins*

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	Percentage of the enrichment (gelatin 100%)†	Moles of amino acid attached/ 100000 g. of gelatin
302, pMetGel	0·55	9·4	9·8	75
207, p(Lys,Glu)Gel {Lys	3·82	8·65	6·6	52
207, p(Lys,Glu)Gel {Glu	10·2	18·3	11·1	86
513, p(Leu,Glu)Gel {Leu	2·98	10·2	9·9	88
513, p(Leu,Glu)Gel {Glu	10·2	16·8	9·05	70

* From Eastoe (1955). † Calculated according to Sela & Arnon (1960*a*).

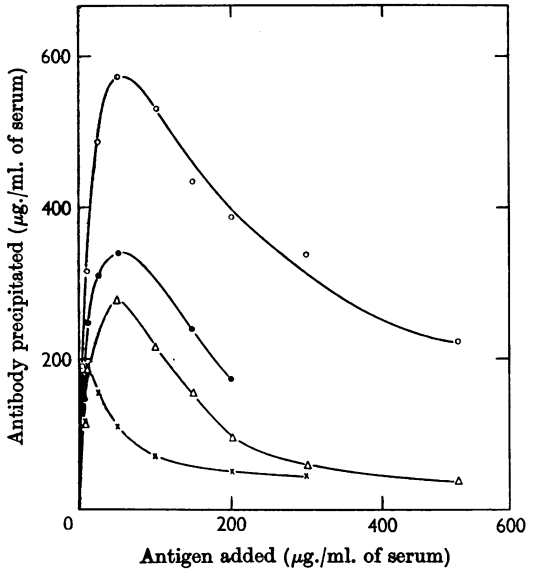

Fig. 1. Precipitin curves of 223, p(Phe,Glu)-pAla--pLys (○), 35, p(Tyr,Glu)-pAla--pLys (●), 34, pGlu-pAla--pLys (△), and 208, pAla--pLys (×), with anti-223. The amount of the antibody was obtained from extinction at 2800 Å.

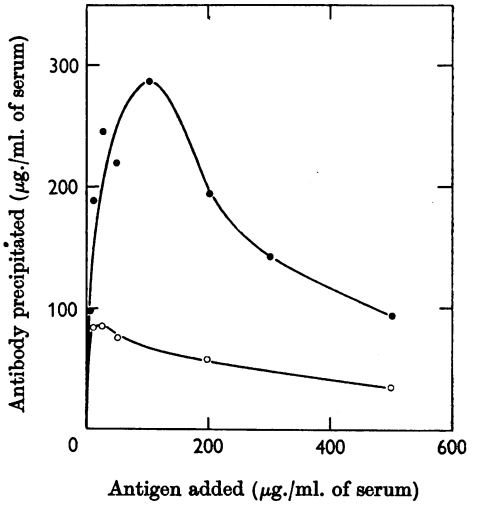

Fig. 2. Precipitin curves of 220, p(His,Glu)-pAla--pLys (●), and 34, pGlu-pAla--pLys (○), with anti-220. The amount of the antibody was obtained from extinction at 2800 Å.

pAla--pLys. Two other derivatives of multichain poly-DL-alanine, 107, p(Leu,Glu)-pAla--pLys, and 203, p(Lys,Glu)-pAla--pLys, cross-precipitated with the antisera to 223, p(Phe,Glu)-pAla--pLys, to the extent of 40 and 30 % of the homologous reaction respectively.

Linear copolymers containing both L-phenylalanine and L-glutamic acid (1:2·1; *n* 110; and 1:4·2; *n* 183), were able to cross-precipitate to the extent of 30 % with antisera to 223. This was in contradistinction to the water-soluble poly-L-glutamic acid, which neither cross-precipitated nor inhibited the homologous reaction of 223, p(Phe,-Glu)-pAla--pLys, as well as to the water-insoluble poly-L-phenylalanine, which did not absorb antibodies against p(Phe,Glu)-pAla--pLys from the antisera.

The antibodies against p(Phe,Glu)-pAla--pLys were characterized also by the haemagglutination test of Boyden (1951). The titre of a typical antiserum was 512. By this technique, the cross-reaction with p(Tyr,Glu)-pAla--pLys could also be detected.

Synthetic polypeptide containing histidine. In view of the unusual chemical structure and some unique properties of the imidazole residue of histidine, it was decided to investigate its potential contribution to immunogenicity. For this purpose, peptides of L-histidine and L-glutamic acid were attached to pAla--pLys, and the resulting 220, p(His,Glu)-pAla--pLys, was injected into rabbits. A good immune response was obtained, as illustrated in Fig. 2. Besides the homologous reaction, the cross-precipitation with pGlu-pAla--pLys is shown in the same Figure. The antisera cross-reacted also with p(Tyr,Glu)-pAla--pLys, p(Leu,-

Glu)-pAla--pLys and pAla--pLys. The titre of the homologous reaction in the haemagglutination test was 256.

Derivatives containing leucine. Leucine was not included in previous experiments in this Laboratory on peptidyl gelatins and synthetic polypeptides. We have now investigated the immunogenicity and antigenic specificity of 513, p(Leu,Glu)Gel, a gelatin derivative enriched with 9·9 % of leucine residues, and 107, p(Leu,Glu)-pAla--pLys, a multichain polypeptide containing 14·2 % of leucine residues. The attachment of peptides of L-leucine and L-glutamic acid to gelatin converted it into a much stronger immunogen (Fig. 3). The antisera cross-precipitated with p(Lys,Glu)Gel (Fig. 3), but neither precipitation nor inhibition was observed with gelatin. This stresses the contribution of glutamic acid, an amino acid which by itself does not enhance the antigenicity of gelatin (Sela & Arnon, 1960*a*), to the antigenic specificity of p(Leu,Glu)Gel. The multichain polypeptide p(Leu,-Glu)-pAla--pLys as well as a linear copolymer of L-leucine and L-glutamic acid [p(Leu,Glu); 1:0·8, *n* 190] did not cross-precipitate with the antibodies to p(Leu,Glu)Gel but inhibited partially the homologous reaction (approx. 25 % at 5 mg. of inhibitor/ml. of serum).

The synthetic 107, p(Leu,Glu)-pAla--pLys, is immunogenic in rabbits (Fig. 4). The cross-reaction with pGlu-pAla--pLys is illustrated in the same Figure. The gelatin derivative, p(Leu,Glu)Gel, inhibited partially the homologous reaction (30 % at 0·5 mg. of inhibitor/ml. of antiserum).

Derivatives with lysine and glutamic acid. Neither poly-L-glutamic acid (Maurer, 1957) nor poly-L-lysine (Maurer, Subrahmanyam, Katchalski & Blout, 1959) are immunogenic. In contrast, some linear copolymers of L-lysine and L-glutamic acid were reported to possess the capacity to elicit antibodies (Gill & Doty, 1961; Maurer, 1962). As the attachment of lysine peptides or glutamic acid peptides to gelatin did not enhance its antigenicity (Sela & Arnon, 1960*a*), it was decided to investigate the contribution of mixed peptides of lysine and glutamic acid to the immunogenic capacity of gelatin.

The sample 207, p(Lys,Glu)Gel, gave a good immune response in rabbits (Fig. 5). The antibodies cross-reacted with p(Lys,Glu)-pAla--pLys (Fig. 5) and cross-precipitated also with p(Leu,Glu)Gel but not with gelatin nor with pTyrGel. A linear copolymer of lysine and glutamic acid inhibited partially the homologous reaction (30 % at 2 mg. of inhibitor/ml. of antiserum).

The immunogenic capacity of peptides of lysine and glutamic acid also becomes apparent on their attachment to the non-immunogenic multichain poly-DL-alanine. The resulting substance was capable of eliciting antibodies, as illustrated in Fig. 5 by the homologous reaction of 203, p(Lys,-Glu)-pAla--pLys. Thus, in contrast with peptides of lysine or of glutamic acid alone, copolypeptides of these two amino acids possess distinct immunogenic features.

The antisera to p(Lys,Glu)-pAla--pLys cross-reacted with p(Lys,Glu)Gel to an extent of 30 % and with pGlu-pAla--pLys only to an extent of 15 %.

Derivatives containing methionine. The attachment of cysteine peptides to gelatin enhanced some-

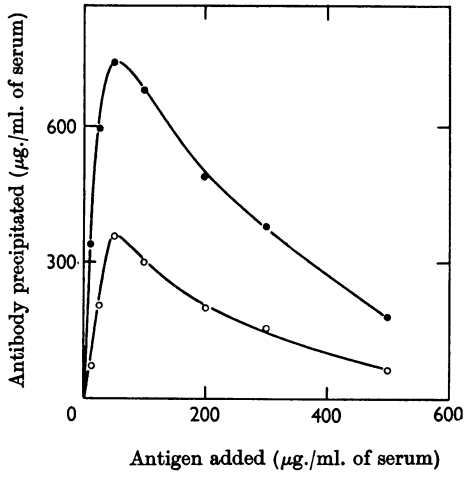

Fig. 3. Precipitin curves of 513, p(Leu,Glu)Gel (●), and 207, p(Lys,Glu)Gel (○), with anti-513. The amount of the antibody was obtained from extinction at 2800 Å.

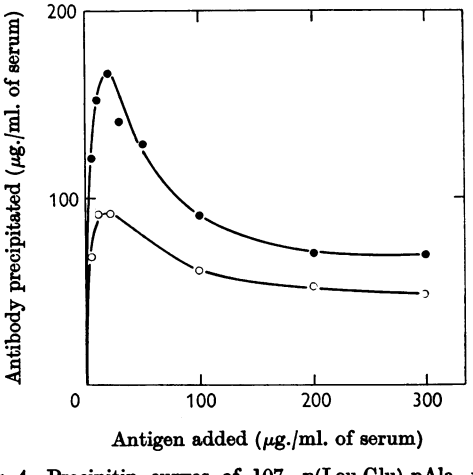

Fig. 4. Precipitin curves of 107, p(Leu,Glu)-pAla--pLys (●), and 34, pGlu-pAla--pLys (○), with anti-107. The amount of the antibody was obtained from extinction at 2800 Å.

what its immunogenicity (Sela & Arnon, 1960*a*), whereas a similar attachment of serine peptides did not convert gelatin into a more powerful antigen (Sela & Arnon, 1960*b*). The investigation of the role of sulphur-containing amino acids in immunogenicity was therefore extended to methionine.

The homologous reaction of a gelatin enriched with 9·8 % of methionine residues, 302, pMetGel, as well as the cross-reaction of anti-pMetGel with pMet-pAla--pLys, are shown in Fig. 6. The antibodies cross-reacted weakly also with gelatin.

Efforts to obtain an immune response towards a multichain synthetic polypeptide containing 10·8 % of methionine residues, 204, pMet-pAla--pLys, were unsuccessful.

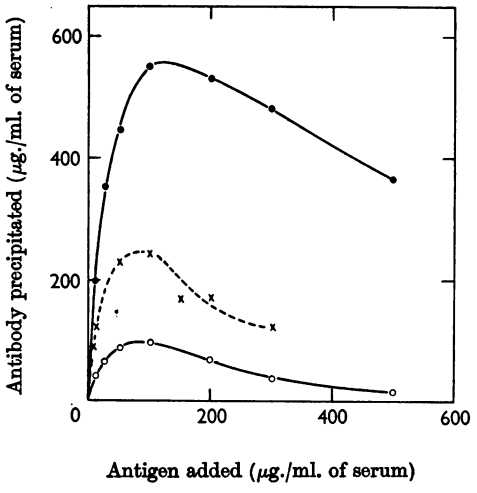

Fig. 5. Precipitin curves of: ●, 207, p(Lys,Glu)Gel, with anti-207; ○, 203, p(Lys,Glu)-pAla--pLys, with anti-207; ×, 203, p(Lys,Glu)-pAla--pLys, with anti-203. The amount of the antibody was obtained from extinction at 2800 Å.

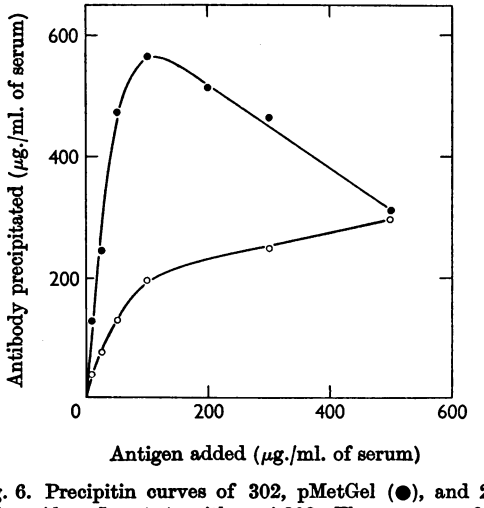

Fig. 6. Precipitin curves of 302, pMetGel (●), and 204, pMet-pAla--pLys (○), with anti-302. The amount of the antibody was obtained from extinction at 2800 Å.

DISCUSSION

The experiments on immunogenicity of synthetic polypeptides and polypeptidyl gelatins tested are summarized in Table 3. The attachment of peptides of glutamic acid and lysine, leucine, histidine or phenylalanine resulted in all cases investigated in the enhancement of the antigenicity of gelatin and in the conversion of the non-antigenic multichain poly-DL-alanine into immunogens. The attachment of methionine peptides enhanced the antigenicity of gelatin, but did not affect multichain poly-DL-alanine.

It may be concluded that the amino acids phenylalanine and histidine, possessing ring structures in their side chains, are immunogenically active, as is tyrosine. The enhancement of gelatin antigenicity upon attachment of methionine peptides would point to the contribution of this amino acid towards immunogenicity, even though its peptides did not convert multichain polyalanine into an immunogen.

The antigens investigated contain determinants of well-defined immune specificity. The amino acids attached contribute in a major way to the antigenic specificity of the immunogens.

The attachment of peptides containing both L-lysine and L-glutamic acid enhanced the antigenicity of gelatin and converted a synthetic non-antigen into an immunogen, in contrast with the results of experiments in which peptides containing only lysine or only glutamic acid were attached. This suggests that diversity, for various reasons, may increase immunogenicity. It is possible that combinations of three or four different amino acids will be able to affect immunogenic properties of macromolecules, even when combinations of the same amino acids (only two at a time) will have no

Table 3. *Immunogenicity of the substances tested*

No. and designation of sample	Antigen added (μg./ml. of serum) in equivalence zone	Antibody precipitated (μg./ml. of serum) in equivalence zone
223, p(Phe,Glu)-pAla--pLys	50	572
220, p(His,Glu)-pAla--pLys	100	270
107, p(Leu,Glu)-pAla--pLys	20	167
203, p(Lys,Glu)-pAla--pLys	100	244
513, p(Leu,Glu)Gel	50	740
207, p(Lys,Glu)Gel	100	550
302, pMetGel	100	562

effect. This may also be one of the reasons why derivatives of gelatin described in this study elicited significantly more antibodies than similar derivatives of the synthetic polymer.

In efforts to understand the structural basis of the immunogenicity and antigenic specificity of proteins it must be remembered that both peptide sequences and areas resulting from the conformation of the molecule, due to a superposition of its secondary, tertiary and quaternary structure, may contribute to immunological properties. For globular proteins the conformational contribution may be even more important than the sequential one. The results reported in this paper and in our previous papers seem, nevertheless, pertinent for the elucidation of the molecular basis of antigenicity of fibrillar proteins and, to a more limited extent, of globular proteins as well.

SUMMARY

1. Several peptidyl derivatives of gelatin and of multichain poly-DL-alanine have been synthesized and characterized. The peptides attached were those of L-methionine as well as copolypeptides of L-glutamic acid and L-phenylalanine, L-histidine, L-leucine or L-lysine.

2. The attachment of peptides of methionine, of leucine and glutamic acid, and of lysine and glutamic acid to gelatin resulted in enhancement of antigenicity, whereas the attachment of copolypeptides of glutamic acid and phenylalanine, histidine, leucine or lysine to multichain polyalanine converted it into immunogens. The attachment of peptides of methionine to multichain polyalanine did not affect its lack of antigenicity.

3. The antigens investigated contain determinants of well-defined immune specificity.

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