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Studies on the Chemical Basis of the Antigenicity of Proteins

6. ANTIGENIC SPECIFICITY OF SOME SYNTHETIC POLYPEPTIDES CONTAINING TYROSINE*

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The main purpose of the investigation of synthetic polypeptide antigens is to determine the minimal structural requirements for immunogenicity, as well as to elucidate their antigenic specificity relationships. The synthesis, characterization and immunogenicity in rabbits of several linear and multichain polypeptides have been described by Sela, Fuchs & Arnon (1962). It was concluded that the immunogenically important area of the molecule must be accessible to the site of the biosynthesis of the antibody, that the overall shape of the molecule does not seem to be an important factor in determining immunogenicity, and that synthetic materials with molecular weights as small as 4000 may be good immunogens.

The present paper is concerned with studies on the specificity of some of the synthetic antigens described previously (Sela & Arnon, 1960*a*; Sela, 1962; Sela *et al.* 1962). In order to elucidate the chemical nature of the determinants of the immune specificity of several synthetic polypeptide antigens, the antisera investigated were allowed to react with a variety of linear and multichain copolymers of amino acids with compositions similar to those of the homologous immunogens as well as with unrelated amino acid compositions. Some proteins and polypeptidyl proteins were also allowed to react with the antisera. Cross-precipitation experiments as well as inhibitions of the homologous reactions were used for this purpose. Similar studies on polypeptidyl derivatives of gelatin have shown that antibodies to polypeptide determinants possess rather narrow specificities (Arnon & Sela, 1960). The present findings show that the synthetic polypeptide antigens investigated contain determinants of well-defined specificity. Thus the antibodies formed against one antigen react well with chemically closely related substances and not at all with unrelated materials.

* Part 5: Sela, Fuchs & Arnon (1962).

EXPERIMENTAL

Materials

The samples of gelatin, ovalbumin, poly-L-tyrosyl ovalbumin, edestin, poly-L-tyrosyl edestin, copolymers of L-tyrosine and L-glutamic acid in residue molar ratios of 1:1·1 (number-average degree of polymerization, *n*, 31), 1:4·0 (*n*, 31) and 1:9·0 (*n*, 88), a copolymer of L-tyrosine and DL-alanine in a residue molar ratio of 1:10·1 (*n*, 35), as well as a copolymer of L-tyrosine, L-leucine and L-glutamic acid in residue molar proportions of 1:1:1 (*n*, 39), were described by Sela & Arnon (1960*b*). The polytyrosyl gelatins, pTyrGel A and pTyrGel B, and copoly-(L-glutamyl,-L-tyrosyl) gelatin, pGluTyrGel, were described by Arnon & Sela (1960).

The synthesis, characterization and nomenclature of the linear and multichain polymers of α-amino acids listed

below were given by Sela *et al.* (1962). The number of the sample precedes the name of the polymer: 102, p(Tyr,Glu); 42, p(Tyr,Glu,Ala); 44, p(Tyr,Glu,Ala); 103, p(Tyr,Glu,-Ala); 3, p(Lys,Ala); 5, pAla--pLys; 19, p(Tyr,Glu)-pAla--p-Lys; 35, p(Tyr,Glu)-pAla--pLys; 33, pTyr-pAla--pLys; 34, pGlu-pAla--pLys; 21, pTyr--pLys; 27, pAla-pTyr--pLys; 31, pGlu-pAla-pTyr--pLys; 22, p(Tyr,Glu)--pLys; 28, pAla-p(Tyr,Glu)--pLys; 106, p(Tyr,Ala)--pLys; 16, pAla-pTyr--p(Lys,Ala); 18, pAla-pTyr--p(Lys,Ala); 26, p(Tyr,Glu)-pAla--p(Lys,Ala), 23, p(Tyr,Glu)--p(Lys,Ala); 30, pAla-p(Tyr,Glu)--p(Lys,Ala); 120, pAla-p(Tyr,Glu)--p-(Lys,Ala).

A sample of poly-L-tyrosyl gelatin containing 16% of tyrosine residues (pTyrGel D) was prepared in a manner analogous to that used for pTyrGel B (Arnon & Sela, 1960). Several multichain copolymers composed of L-lysine, L-tyrosine, L-glutamic acid and/or DL-alanine were prepared and analysed according to the methods described by Sela *et al.* (1962), and are listed in Table 1.

Immune sera

The preparation of the immune sera used in this study was described by Sela *et al.* (1962). They included antisera to: 33, pTyr-pAla--pLys; 19, p(Tyr,Glu)-pAla--pLys; 35, p(Tyr,Glu)-pAla--pLys; 22, p(Tyr,Glu)--pLys; 30, pAla-p-(Tyr,Glu)--p(Lys,Ala); 102, p(Tyr,Glu); 42, p(Tyr,Glu,Ala); 44, p(Tyr,Glu,Ala). Thiomersal (final concn. 0·01%) was added to all sera.

Labelling of antigens

Samples 22, p(Tyr,Glu)--pLys, and 23, p(Tyr,Glu)--p-(Lys,Ala), were iodinated with [^{131}I]iodide (50 μc/10 mg.) as described by Talmage, Baker & Akeson (1954). The substances, isolated after exhaustive dialysis, had radioactivities in the range 500 000–1 000 000 counts/min./mg.

Methods

Precipitin studies. All experiments were carried out with pooled antisera. Before cross-reaction experiments, all the homologous and heterologous materials used in this study were allowed to react with normal rabbit sera. None of the materials gave any precipitate, except for three multichain polymers containing tyrosine but no glutamic acid (molecules with overall positive charge at neutral pH values). These polymers [21, pTyr--pLys; 33, pTyr-pAla--pLys; 106, p(Tyr,Ala)--pLys] caused some precipitation when added in amounts higher than 0·2 mg./ml. of normal serum.

In all cases, increasing amounts of the homologous immunogen, or of the heterologous materials tested for cross-reactions with the antiserum, were dissolved in equal volumes of aq. 0·9% sodium chloride and added to test tubes containing a constant volume of serum (0·2, 0·5 or 1·0 ml., depending on the immune response). The final volume never exceeded 1·5 ml. In control experiments aq. 0·9% sodium chloride was added instead of the solution of the substance tested. 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.

For quantitative precipitin tests, the tubes were centrifuged, and the precipitates that formed were washed three times with chilled (2°) aq. 0·9% sodium chloride and dissolved in 0·1 N-sodium hydroxide (1·1 ml.). The extinction of these solutions was read at 2800 Å. The amount of antigen in some precipitates was determined after labelling with ^{131}I. The antibody content was then obtained from the measured extinction after deducting the calculated extinction of the antigen.

Inhibition studies. Whenever no cross-precipitation was observed within 48 hr., homologous antigen was added in the optimum amount and the precipitin test was carried out as described above. Any decrease in the amount of immune precipitate was considered to be an indication of an inhibitory effect by the cross-reactant.

Quantitative inhibition studies were performed as follows: Substances checked as inhibitors of the homologous precipitin reactions were added in increasing concentrations to 0·5 ml. of the antiserum. After an incubation of 30 min. at 37° an amount of homologous 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. Any precipitates that formed were investigated quantitatively as described above.

Radioactivity. The 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 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.

RESULTS

The antisera used in this study were allowed to react with materials closely related chemically to the homologous antigens as well as with unrelated substances. Some of the positive immunospecific cross-precipitations were investigated quantitatively. Whenever no cross-precipitations were observed, the materials checked were investigated

Table 1. *Multichain copolymers of α-amino acids*

The partial specific volumes, $\bar{v}$, of the copolymers were calculated as described by Sela *et al.* (1962). Other details are given in the text.

No. and designation of sample	Molar ratio of amino acid residues in the copolymer				$\bar{v}$ (ml./g.)	$S_{20,w}$ (s)	$10^7 D_{20,w}$ (cm.^{2}/sec.)	Average mol. wt.
	L-Lys	L-Tyr	L-Glu	DL-Ala				
36, pGlu-pTyr-pAla--pLys	1·0	1·7	2·17	24	0·70	2·92	6·1	38 800
37, pTyr-pGlu-pAla--pLys	1·0	1·0	3	22	0·70	2·87	5·1	45 500
112, p(Tyr,Glu)--pLys	1·0	1·1	2·3	—	0·65	2·25	10·6	14 700

for their possible inhibitory capacity. None of the antisera investigated reacted with proteins such as ovalbumin, edestin or gelatin.

Antigenic specificity of pTyr-pAla--pLys

Both tyrosine and alanine contributed to the antigenic specificity of pTyr-pAla--pLys. The role of tyrosine in the antigenic specificity of this immunogen is apparent from the fact that, with one exception (pAla--pLys), all the materials that cross-reacted with anti-pTyr-pAla--pLys contained at least 5% of tyrosine. Thus the following substances gave immunospecific precipitates with antisera to pTyr-pAla--pLys: pTyrGel A (10% of tyrosine); pTyrGel D (16% of tyrosine); 21, pTyr--pLys; 16, pAla-pTyr--p(Lys,Ala); 35, p(Tyr,Glu)-pAla--pLys; 28, pAla-p(Tyr,Glu)--p-Lys; 30, pAla-p(Tyr,Glu)--p(Lys,Ala). All the above materials gave a significant precipitation at the lowest amount checked (100 μg./ml. of antiserum). Substances that did not give cross-precipitation but inhibited the homologous reaction of this antiserum are listed in Table 2. Neither precipitation nor inhibition was observed when 34, pGlu-pAla--pLys, or 22, p(Tyr,Glu)--pLys, was added.

The role of the extent of tyrosine content in the material that had reacted with the antiserum is illustrated by experiments with gelatin derivatives. Those enriched with 10 or 16% of tyrosine residues gave cross-precipitation in this system, whereas the derivative with only 5% of tyrosine residues inhibited the homologous reaction.

The importance of alanine in the specificity site of pTyr-pAla--pLys is stressed by the interaction of the antibodies with pAla--pLys, a material that served as the multifunctional initiator in the synthesis of the immunogen pTyr-pAla--pLys. The presence of glutamic acid in the molecule appears to interfere in the reaction with antiserum to pTyr-pAla--pLys. Thus neither p(Tyr,Glu)--pLys nor pGlu-pAla--pLys reacts, in contrast with pTyr--pLys, which gives cross-precipitation with antibodies to pTyr-pAla--pLys, and pAla--pLys, which inhibits the homologous reaction.

Antigenic specificity of p(Tyr,Glu)-pAla--pLys

The antisera to p(Tyr,Glu)-pAla--pLys gave cross-precipitation with all materials tested that contained copolypeptides of tyrosine and glutamic acid on the outside of the molecule [pTyrGluGel; 112, p(Tyr,Glu)--pLys; 23, p(Tyr,Glu)--p(Lys,Ala); 26, p(Tyr,Glu)-pAla--p(Lys,Ala)]. Precipitation with 112, p(Tyr,Glu)--pLys, is shown in Fig. 1. The cross-reaction with pTyrGluGel was reported by Sela & Arnon (1960*a*). The linear copolymers 102, p(Tyr,Glu), and 44, p(Tyr,Glu,Ala), also gave good precipitation with the antiserum to 35, p(Tyr,-Glu)-pAla--pLys (Fig. 1).

Table 2. *Inhibition of the precipitation of the system 33–anti-33 (33 is pTyr-pAla--pLys)*

The amount of homologous antigen added was 50 μg./ml. of antiserum. + + +, Complete inhibition; + +, strong inhibition; +, slight inhibition; −, no inhibition.

Inhibitor	Amount added (μg./ml. of serum)	Inhibition
Poly-L-tyrosyl ovalbumin	1000	+
	5000	+ + +
Poly-L-tyrosyl edestin	1000	+
	5000	+ +
pTyrGel B (5% of tyrosine)	1000	−
	5000	+
27, pAla-pTyr--pLys	250	+ +
	1000	+ +
	5000	+ + +
p(Tyr,Ala) (1:10)	250	+ +
	1000	+ + +
	5000	+ + +
37, pTyr-pGlu-pAla--pLys	1000	+ +
	5000	+ + +
36, pGlu-pTyr-pAla--pLys	250	+ +
	1000	+ + +
	5000	+ + +
44, p(Tyr,Glu,Ala)	1000	−
	5000	+ +
5, pAla--pLys	1000	+ +
	2000	+ + +

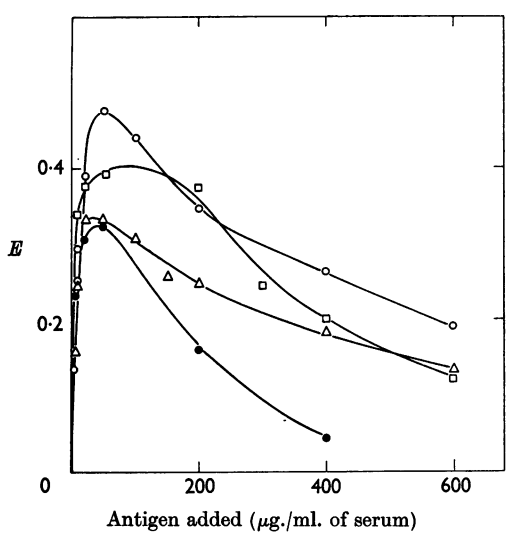

Fig. 1. Extinction at 2800 Å of solutions in 0·1 N-sodium hydroxide of precipitates obtained by the addition to an antiserum to 35, p(Tyr,Glu)-pAla--pLys, of: ○, 35, p(Tyr,Glu)-pAla--pLys; □, 44, p(Tyr,Glu,Ala); △, 112, p(Tyr,Glu)--pLys; ●, 102, p(Tyr,Glu).

Two multichain macromolecules in which tyrosine and glutamic acid are present as peptides attached directly to the polymer backbone and covered by polyalanine side chains 30, pAla-p(Tyr,-Glu)--p(Lys,Ala), and 28, pAla-p(Tyr,Glu)--pLys, gave cross-precipitation with the antisera discussed (Fig. 2). Precipitation was also observed with 36, pGlu-pTyr-pAla--pLys, and 37, pTyr-pGlu-p-Ala--pLys. In both these polymers tyrosine and glutamic acid are present as peptide blocks and not distributed randomly.

The following multichain polymers containing tyrosine and alanine, but no glutamic acid, gave significant precipitin reactions with antiserum to 35: 106, p(Tyr,Ala)--pLys; 33, pTyr-pAla--pLys; 16, pAla-pTyr--p(Lys,Ala).

The homologous reaction of the system

p(Tyr,Glu)-pAla--pLys–
anti-p(Tyr,Glu)-pAla--pLys

could be inhibited completely by a copolymer of L-tyrosine and L-glutamic acid in a residue molar ratio 1:1·1 (*n*, 31), but only partially by a copolymer of these amino acids in a ratio 1:4 (*n*, 31). 34, pGlu-pAla--pLys, inhibited only partially the 35–anti-35 system at the highest concentration checked (5000 μg./ml.), but the inhibition by 31, pGlu-pAla-pTyr--pLys, was complete at the same concentration and partial inhibition was already observed at 1000 μg./ml. Neither precipitation nor inhibition was observed when pTyrGel B (5 % of tyrosine), pTyrGel D (16 % of tyrosine), a copolymer of L-tyrosine and L-glutamic acid (1:9; *n*, 88), or 27, pAla-pTyr--pLys, was added.

The cross-reactions in this system, as in that with pTyr-pAla--pLys, are specific as non-related proteins were completely negative. Though it is not surprising that materials containing both tyrosine and glutamic acid cross-reacted with antisera to p(Tyr,Glu)-pAla--pLys, it is interesting that cross-precipitation was observed both with the immunogenic 30, pAla-p(Tyr,Glu)--p(Lys,Ala), and with the non-immunogenic 28, pAla-p(Tyr,Glu)--pLys. On the other hand, in the absence of glutamic acid, 16, pAla-pTyr--p(Lys,Ala), gave cross-reaction, though 27, pAla-pTyr--pLys, did not. The attachment of glutamic acid converted pAla-pTyr--pLys into a cross-reacting material.

Antigenic specificity of p(Tyr,Glu)--pLys

The antisera to p(Tyr,Glu)--pLys reacted exclusively with materials containing both tyrosine and glutamic acid. The cross-precipitation with 23, p(Tyr,Glu)--p(Lys,Ala), as well as the homologous precipitin curve, are given in Fig. 3. The antigens were in this case trace-iodinated with ^{131}I. Reactions with pTyrGluGel and 35, p(Tyr,Glu)-pAla--p-Lys, are shown in Fig. 4; those with the linear polymers 102, p(Tyr,Glu), 42, p(Tyr,Glu,Ala),

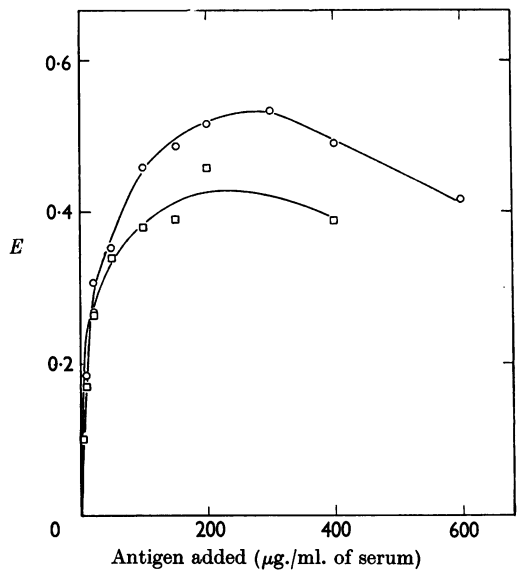

Fig. 2. Extinction at 2800 Å of solutions in 0·1 N-sodium hydroxide of precipitates obtained by the addition to an antiserum to 35, p(Tyr,Glu)-pAla--pLys, of: ○, 30, pAla-p(Tyr,Glu)--p(Lys,Ala); □, 28, pAla-p(Tyr,Glu)--p-Lys.

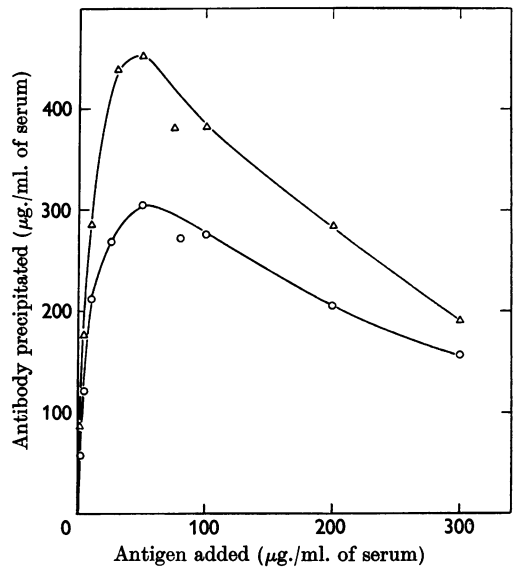

Fig. 3. Precipitin curves of 22, p(Tyr,Glu)--pLys (△), and 23, p(Tyr,Glu)--p(Lys,Ala) (○), with anti-22. The amount of antibody was obtained from the extinction at 2800 Å after deducting the calculated extinction of the antigen. The amount of antigen in the precipitate was obtained from radioactivity data.

44, p(Tyr,Glu,Ala), and 103, p(Tyr,Glu,Ala), are shown in Fig. 5. The polymers 26, p(Tyr,Glu)-pAla--p(Lys,Ala), 28, pAla-p(Tyr,Glu)--pLys, and 37, pTyr-pGlu-pAla--pLys, were also precipitated with this antiserum.

The inhibition of the homologous reaction by a copolymer of L-tyrosine and L-glutamic acid (1:1·1; *n*, 31) as well as by a copolymer of L-tyrosine, L-leucine and L-glutamic acid (1:1:1; *n*, 39) is shown in Fig. 6. In both cases almost complete inhibition may be reached. A 50% inhibition of maximal precipitation was obtained at a ratio of inhibitor to homologous antigen of 31:1 (w/w) or 70:1 (molar ratio) for the copolymer of L-tyrosine and L-glutamic acid and 48:1 (w/w) or 94:1 (molar ratio) for the copolymer of L-tyrosine, L-leucine and L-glutamic acid. The ratio of inhibitor to precipitating antibody was 2·2:1 (w/w) and 3·4:1 (w/w) for the two copolymers respectively. The precipitation was also inhibited by 30, pAla-p(Tyr,Glu)--p(Lys,-Ala), at 5000 μg./ml. and by a copolymer of L-tyrosine and L-glutamic acid (1:4) at 1000 μg./ml. Only slight inhibition was obtained at 5000 μg./ml. with a copolymer of L-tyrosine and L-glutamic acid (1:9).

The antisera to p(Tyr,Glu)--pLys did not react with the following substances in which either tyrosine or glutamic acid is absent: pTyrGel D (16% of tyrosine); polytyrosyl edestin; 33, pTyr-p-Ala--pLys; 34, pGlu-pAla--pLys; 27, pAla-p-Tyr--pLys; a copolymer of L-tyrosine and DL-alanine (1:10). 33 and 34 did not react when mixed together. The only substance with both glutamic acid and tyrosine that did not cross-react with the

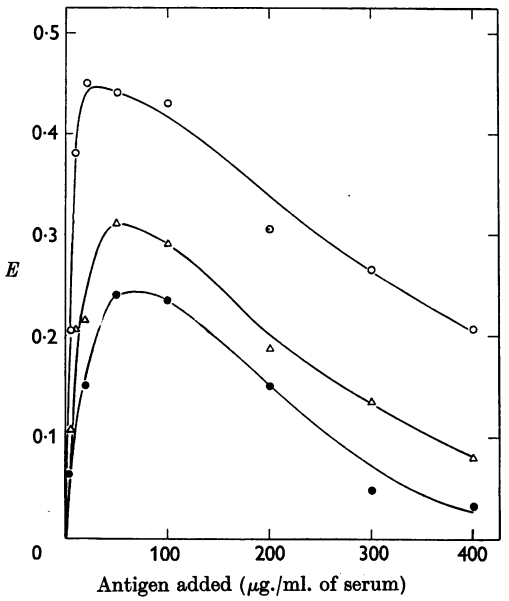

Fig. 4. Extinction at 2800 Å of solutions in 0·1 N-sodium hydroxide of precipitates obtained by the addition to an antiserum to 22, p(Tyr,Glu)--pLys, of: ○, 22, p(Tyr,-Glu)--pLys; △, 35, p(Tyr,Glu)-pAla--pLys; ●, pTyrGluGel.

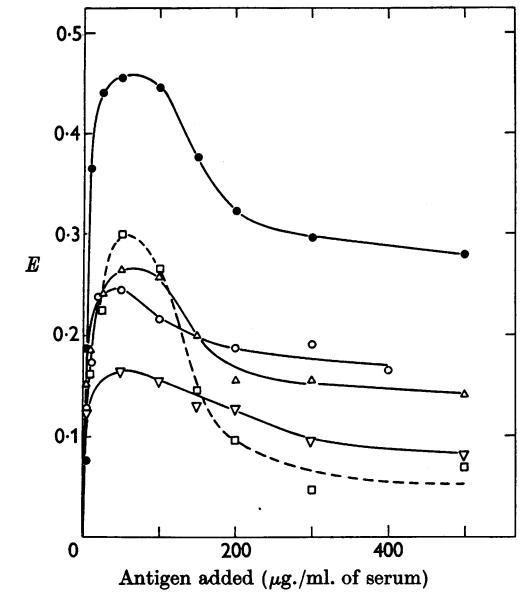

Fig. 5. Extinction at 2800 Å of solutions in 0·1 N-sodium hydroxide of precipitates obtained by the addition to an antiserum to 22, p(Tyr,Glu)--pLys, of: ●, 22, p(Tyr,-Glu)--pLys; □, 44, p(Tyr,Glu,Ala); △, 103, p(Tyr,Glu,Ala); ○, 102, p(Tyr,Glu); ▽, 42, p(Tyr,Glu,Ala).

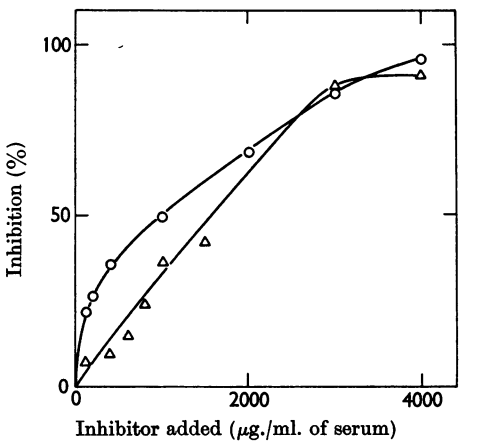

Fig. 6. Inhibition curves of the homologous reaction of the system 22–anti-22 [22 is p(Tyr,Glu)--pLys] with: ○, a copolymer of L-tyrosine and L-glutamic acid(1:1·1; *n*, 31); △, a copolymer of L-tyrosine, L-leucine and L-glutamic acid (1:1:1; *n*, 39). The amount of homologous antigen added was 35 μg./ml. of antiserum.

antisera discussed was 36, pGlu-pTyr-p-Ala--pLys, in which a block peptide of glutamic acid is attached to a block peptide of tyrosine.

Antigenic specificity of pAla-p(Tyr,Glu)--p(Lys,Ala)

The multichain polymers 30 and 120, of the formula pAla-p(Tyr,Glu)--p(Lys,Ala), are immunogenic even though they contain tyrosine on the inside of the molecule (Sela *et al.* 1962). This was interpreted as being due to the increased average distance between the polypeptide side chains within the molecule as compared with the non-immunogenic 28, pAla-p(Tyr,Glu)--pLys. In the present work 28, pAla-p(Tyr,Glu)--pLys, gave good cross-precipitation with antisera to pAla-p(Tyr,-Glu)--p(Lys,Ala), and so did, in a more limited way, 27, pAla-pTyr--pLys (Fig. 7). The cross-precipitations with 35, p(Tyr,Glu)-pAla--pLys, and with 16, pAla-pTyr--p(Lys,Ala), are also given in Fig. 7. Some precipitation was also obtained with the following polymers: 5, pAla--pLys; 26, p(Tyr,-Glu)-pAla--p(Lys,Ala); 37, pTyr-pGlu-pAla--pLys; 18, pAla-pTyr--p(Lys,Ala).

Inhibitors of the homologous reaction of this antiserum are listed in Table 3. Neither precipitation nor inhibition was observed with pTyrGel B (5% of tyrosine), pTyrGluGel or 22, p(Tyr,-Glu)--pLys.

With one exception, namely 102, p(Tyr,Glu), all materials that reacted with the antisera discussed contained alanine, demonstrating the importance of this amino acid for the antigenic specificity of pAla-p(Tyr,Glu)--p(Lys,Ala). On the other hand two substances containing tyrosine and glutamic acid but no alanine were completely unreactive.

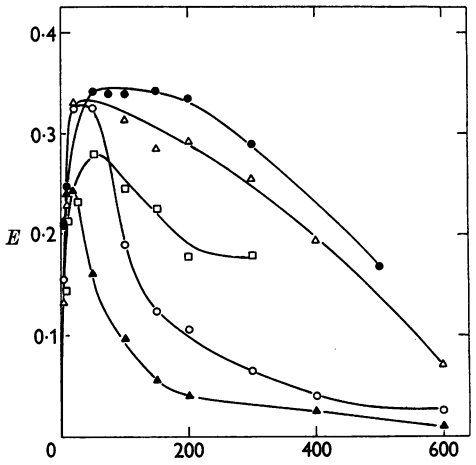

Fig. 7. Extinction at 2800 Å of solutions in 0·1 N-sodium hydroxide of precipitates obtained by the addition to an antiserum to 30, pAla-p(Tyr,Glu)--p(Lys,Ala), of: ●, 30, pAla-p(Tyr,Glu)--p(Lys,Ala); △, 28, pAla-p(Tyr,Glu)--p-Lys; ○, 35, p(Tyr,Glu)-pAla--pLys; □, 16, pAla-pTyr--p-(Lys,Ala); ▲, 27, pAla-pTyr--pLys.

Table 3. *Inhibition of the precipitation of the system 30–anti-30 [30 is pAla-p(Tyr,Glu)--p(Lys,Ala)]*

The amount of homologous antigen added was 75 μg./ml. of antiserum. + + +, Complete inhibition; + +, strong inhibition; +, slight inhibition.

Inhibitor	Amount added (μg./ml. of serum)	Inhibition
3, p(Lys,Ala)	1000	+
	5000	+ + +
23, p(Tyr,Glu)--p(Lys,Ala)	1000	+
	5000	+ +
31, pGlu-pAla-pTyr--pLys	250	+
	1000	+ +
	5000	+ + +
102, p(Tyr,Glu)	1000	+
	5000	+

Antigenic specificity of p(Tyr,Glu)

As with the antisera to p(Tyr,Glu)--pLys, those to p(Tyr,Glu) reacted only with substances containing both tyrosine and glutamic acid. Fig. 8

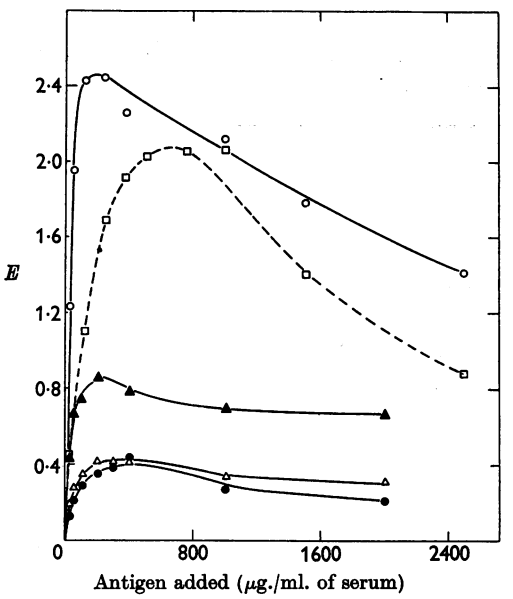

Fig. 8. Extinction at 2800 Å of solutions in 0·1 N-sodium hydroxide of precipitates obtained by the addition to an antiserum to 102, p(Tyr,Glu), of: ○, 102, p(Tyr,Glu); □, 44, p(Tyr,Glu,Ala); ▲, 112, p(Tyr,Glu)--pLys; △, 19, p(Tyr,Glu)-pAla--pLys; ●, 30, pAla-p(Tyr,Glu)--p(Lys,-Ala.)

shows the cross-precipitations of the antiserum to 102, p(Tyr,Glu), with the following: 44, p(Tyr,Glu,-Ala); 112, p(Tyr,Glu)--pLys; 19, p(Tyr,Glu)-p-Ala--pLys; 30, pAla-p(Tyr,Glu)--p(Lys,Ala). The polymers 103, p(Tyr,Glu,Ala), 42, p(Tyr,Glu,Ala), and 35, p(Tyr,Glu)-pAla--pLys, as well as a copolymer of L-tyrosine and L-glutamic acid of a low molecular weight (1:1·1; *n*, 31), also precipitated antibodies to p(Tyr,Glu).

Inhibition of the homologous reaction was observed with pTyrGluGel (5000 μg./ml.), 28, pAla-p(Tyr,Glu)--pLys (500 μg./ml.), a copolymer of L-tyrosine, L-leucine and L-glutamic acid (1:1:1; *n*, 39) (1000 μg./ml.), and a copolymer of L-tyrosine and L-glutamic acid (1:9; *n*, 88). Neither precipitation nor inhibition was found with a copolymer of L-tyrosine and DL-alanine (1:10; *n*, 35), with 33, pTyr-pAla--pLys, with 34, pGlu-pAla--pLys, or with 27, pAla-pTyr--pLys.

Antigenic specificity of p(Tyr,Glu,Ala)

Antisera to 42, p(Tyr,Glu,Ala), and 44, p(Tyr,-Glu,Ala), two polymers containing L-alanine, gave precipitation reactions only with 42, 44, and also 103, p(Tyr,Glu,Ala), a polymer containing DL-alanine (Fig. 9).

For the system 42–anti-42 inhibitions were obtained only with 102, p(Tyr,Glu), and a copolymer of L-tyrosine, L-leucine and L-glutamic acid (1:1:1; *n*, 39). In the system 44–anti-44 inhibition was found also with 35, p(Tyr,Glu)-pAla--pLys, 26, p(Tyr,Glu)-pAla--p(Lys,Ala), and 37, pTyr-pGlu-p-Ala--pLys. The slightly different behaviour of the two antisera may be due to the difference in molecular weight and in the tyrosine content of the two immunogens.

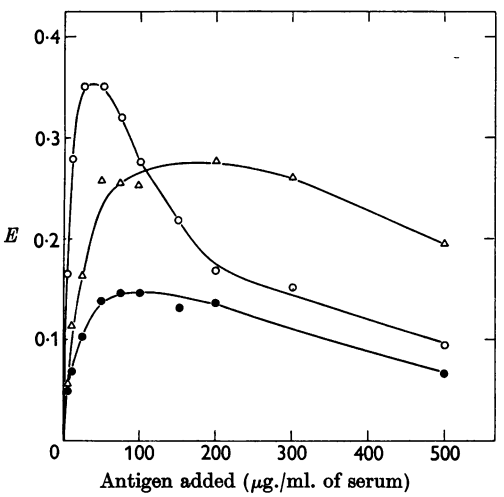

Fig. 9. Extinction at 2800 Å of solutions in 0·1 N-sodium hydroxide of precipitates obtained by the addition to an antiserum to 42, p(Tyr,Glu,Ala), of: ○, 42, p(Tyr,Glu,Ala); △, 44, p(Tyr,Glu,Ala); ●, 103, p(Tyr,Glu,Ala).

No precipitation or inhibition was obtained with either of the antisera on reaction with the following: pTyrGel B (5 % of tyrosine) and pTyrGel D (16 % of tyrosine); pTyrGluGel; a copolymer of L-tyrosine and L-glutamic acid (1:9; *n*, 88); 28, pAla-p(Tyr,Glu)--pLys; 30, pAla-p(Tyr,Glu--p-(Lys,Ala); 112, p(Tyr,Glu)--pLys. Antisera to p(Tyr,Glu,Ala) reacted with the linear polymer p(Tyr,Glu) but not with the multichain polymer p(Tyr,Glu)--pLys.

DISCUSSION

Sela *et al.* (1962) reported that, though tyrosine is not unique in conferring immunogenicity, with the polymers investigated the presence of tyrosine was necessary to render them immunogenic. In the present paper the contribution of the component amino acids to the antigenic specificity of some immunogens containing tyrosine as well as glutamic acid, alanine and/or lysine is described. From the results reported it may be concluded that tyrosine contributes to the antigenic specificity of the immunogens in which it is present, and so do other amino acids (e.g. glutamic acid or alanine) in the molecule. Most of the cross-precipitation and inhibition experiments are summarized in Table 4. It is apparent that antibodies formed in rabbits against the synthetic polypeptide antigens investigated possess combining sites of well-defined immune specificity. Similar conclusions on the specificity of synthetic polypeptide antigens may be reached from the studies of Gill & Doty (1961, 1962), of Maurer (1962) and of Maurer, Gerulat & Pinchuck (1962).

The nature of the antigenic specificity of the immunogens investigated may be deduced from the experiments described. Thus, for example, in two polymers, p(Tyr,Glu) and p(Tyr,Glu)--pLys, the immune determinant is composed of tyrosine and glutamic acid. Antibodies to both these materials reacted exclusively with substances containing peptides of tyrosine and glutamic acid. For p(Tyr,Glu)-pAla--pLys the specificity was directed mainly to peptides of tyrosine and glutamic acid, but alanine participated in this case in the antigenic-specificity site, as illustrated by cross-precipitations of the antiserum with pTyr-pAla--pLys, p(Tyr,Ala)--pLys and pAla-pTyr--p(Lys,Ala), as well as partial inhibition with pGlu-pAla--pLys.

The contribution of alanine to specificity is also apparent from the lack of reaction of anti-p(Tyr,-Glu,Ala) with several materials containing tyrosine and glutamic acid but no alanine. In pTyr-pAla--p-Lys both tyrosine and alanine participate in the antigenic specificity. Antibodies reacted not only

with substances containing both tyrosine and alanine but also with some materials that contained tyrosine but no alanine (pTyr--pLys) or alanine but no tyrosine (pAla--pLys).

Multichain polymers in which peptides of tyrosine or of tyrosine and glutamic acid are attached directly to polylysine and are covered by polyalanine side chains [27, pAla-pTyr--pLys; 28, pAla-p(Tyr,-Glu)--pLys] are not immunogenic (Sela *et al.* 1962). On the other hand, multichain polymers in which the average distance between the polymeric side chains was increased considerably by attaching the tyrosine-containing peptides, still covered by polyalanine, to copolymers of lysine and alanine [18, pAla-pTyr--p(Lys,Ala); 30, pAla-p(Tyr,Glu)--p(Lys,Ala)] are immunogenic. One of the main conclusions of the present paper is that substances which are not immunogenic, because the area important for immunogenicity is not accessible to the biosynthetic site, may nevertheless cross-react with antibodies formed against substances closely related chemically. Thus the non-immunogenic pAla-p(Tyr,Glu)--pLys (Sela *et al.* 1962) reacts well with anti-30 [anti-pAla-p(Tyr,Glu)--p(Lys,Ala)], as well as with several other antisera, and pAla-pTyr--pLys inhibits the reaction between pTyr-pAla--pLys and anti-pTyr-pAla--pLys. Apparently, the capacity of a molecule to react with the combining site of an antibody is not parallel with its ability to elicit the formation of antibodies.

Table 4. *Cross-reactivity of antisera against several synthetic immunogens*

H, homologous precipitation; P, cross-precipitation; I, inhibition; —, no reaction.

Materials tested	Antisera against: pTyr-pAla--pLys	p(Tyr,Glu)-pAla--pLys	p(Tyr,Glu)--pLys	pAla-p(Tyr,Glu)--p(Lys,Ala)	102, p(Tyr,Glu)	p(Tyr,Glu,Ala)
pTyrGel B	I	—	.	—	.	—
pTyrGel D	P	—	—	.	.	—
Polytyrosyl edestin	I	.	—	.	.	.
pTyrGluGel	.	P	P	—	I	—
102, p(Tyr,Glu)	.	P	P	I	H	I
p(Tyr,Glu) (1:1·1; *n*, 31)	.	I	I	.	P	.
p(Tyr,Glu) (1:4; *n*, 31)	.	I	I	.	.	.
p(Tyr,Glu) (1:9; *n*, 88)	.	—	I	.	I	—
p(Tyr,Leu,Glu) (1:1:1; *n*, 39)	.	.	I	.	I	I
42, p(Tyr,Glu,Ala)	.	.	P	.	P	H
44, p(Tyr,Glu,Ala)	I	P	P	.	P	H
103, p(Tyr,Glu,Ala)	.	.	P	.	P	P
p(Tyr,Ala) (1:10·1; *n*, 35)	I	.	—	.	—	.
3, p(Lys,Ala)	.	.	.	I	.	.
5, pAla--pLys	I	.	.	P	.	.
21, pTyr--pLys	P	.	.	.	.	.
106, p(Tyr,Ala)--pLys	P	.	.	.	.	.
33, pTyr-pAla--pLys	H	P	—	.	—	.
34, pGlu-pAla--pLys	—	I	—	.	—	.
35, p(Tyr,Glu)-pAla--pLys	P	H	P	P	P	I
26, p(Tyr,Glu)-pAla--p(Lys,Ala)	.	P	P	P	.	I
112, p(Tyr,Glu)--pLys	.	P	.	.	P	—
22, p(Tyr,Glu)--pLys	—	.	H	—	.	.
23, p(Tyr,Glu)--p(Lys,Ala)	.	P	P	I	.	.
36, pGlu-pTyr-pAla--pLys	I	P	—	.	.	.
37, pTyr-pGlu-pAla--pLys	I	P	P	P	.	I
31, pGlu-pAla-pTyr--pLys	.	I	.	I	.	.
27, pAla-pTyr--pLys	I	—	—	P	—	.
16, pAla-pTyr--p(Lys,Ala)	P	P	.	P	.	.
18, pAla-pTyr--p(Lys,Ala)	.	.	P	.	.	.
28, pAla-p(Tyr,Glu)--pLys	P	P	P	P	I	—
30, pAla-p(Tyr,Glu)--p(Lys,Ala)	P	P	.	H	P	—

It seems that the specificity is governed by the area of the molecule most exposed to the surroundings. Thus tyrosine and glutamic acid are more important for the specificity of p(Tyr,Glu)-pAla--p-Lys, and alanine becomes of greater importance in pAla-p(Tyr,Glu)--p(Lys,Ala). Antisera to 30, pAla-p(Tyr,Glu)--p(Lys,Ala), reacted not only with materials containing tyrosine and alanine but also with polymers containing alanine and no tyrosine. The involvement of alanine in this system is clearly indicated by the difference in the reactivity of p(Tyr,Glu)--pLys and p(Tyr,Glu)-p(Lys,Ala). The polymer containing alanine inhibited the homologous reaction, but p(Tyr,Glu)--pLys did not react at all with anti-30.

Antibodies to linear polypeptide antigens were precipitated much better with linear than with multichain polymers. This is seen in Fig. 8 and Table 4.

The role of molecular weight in the type of immunospecific reaction obtained is illustrated by the difference in the behaviour of two polymers of identical composition but different molecular weights in the systems 22–anti-22 [22 is p(Tyr,-Glu)--pLys] and 35–anti-35 [35 is p(Tyr,Glu)-p-Ala--pLys]. The immunogenic copolymer 102, p(Tyr,Glu) (in a residue molar ratio 1:1), of number-average molecular weight 12 500, gave cross-precipitation with the antisera in both cases (Figs. 1 and 5). In contradistinction, a copolymer of L-tyrosine and L-glutamic acid (in a residue molar ratio 1:1·1; n, 31), of number-average molecular weight 4500, which did not elicit antibodies when injected into rabbits (Sela & Arnon, 1960*a*), caused in these systems inhibition of the homologous reaction (Fig. 6) but gave no cross-precipitation.

The linear copolymers used in the present study possess a random sequence. Thus copolymers of L-tyrosine and L-glutamic acid in approximately equimolar residue ratio contain in their chains many regions of repeating units of tyrosine or of glutamic acid. Sequences of adjacent tyrosine residues may probably still occur in copolymers of tyrosine and glutamic acid in a residue molar ratio of 1:4, but not in those with a ratio of 1:9. It is interesting to compare the inhibitory efficiency of these copolymers in immunospecific reactions. Sela & Arnon (1960*b*) observed that only polymers with a high content of tyrosine inhibited well the system pTyrGel–anti-pTyrGel, and they concluded that sequences of several tyrosine residues are necessary for the inhibition. With pTyrGluGel, however, both tyrosine and glutamic acid contribute to its specificity. Thus only a copolymer with a residue molar ratio of these two amino acids similar to their ratio in the gelatin derivative reacted with the antisera (Arnon & Sela, 1960). The present study corroborates this finding. Thus in the p(Tyr,Glu)-p-Ala--pLys–anti-p(Tyr,Glu)-pAla--pLys system the copolymer of tyrosine and glutamic acid in a ratio of 1:1·1 inhibited completely the homologous reaction, the copolymer in a ratio of 1:4 inhibited it only partially, and the copolymer in a ratio of 1:9 did not react at all with the antisera. A similar situation was obtained with the system p(Tyr,Glu)--pLys–anti-p(Tyr,Glu)--pLys.

The inhibitions of the system 22–anti-22 [22 is p(Tyr,Glu)--pLys] by the copolymer p(Tyr,Glu) (1:1; n, 31), as well as by a copolymer containing leucine in addition to tyrosine and glutamic acid, are given in Fig. 6. Almost complete inhibition was obtained in both cases. The failure to get complete inhibition of precipitation of an antigen with its antiserum by inhibitors derived from the original antigen has been interpreted as being due to the fact that the antisera are mixtures of antibodies directed against different antigenic sites on the immunogen (Porter, 1957; Press & Porter, 1962). Conversely, the ability to inhibit almost totally the homologous reaction in the p(Tyr-Glu)--pLys–anti-p(Tyr,Glu)--pLys system with linear copolymers of a relatively low molecular weight and high content of tyrosine and glutamic acid seems to imply that there is only one type of antigenic site on the synthetic immunogen p(Tyr,Glu)--pLys. The molar ratios of the inhibitors to the immunogen at 50 % of the total inhibition were in the above case 70 and 94, and these values should be compared with those (0·3–1·5) obtained by Press & Porter (1962) at 50 % of the maximum inhibition by fragments of human albumin on the one hand, and those (1000–3000) obtained with haptens (Beiser, Burke & Tanenbaum, 1960), polyglucose chains (Kabat, 1960) and peptides from silk fibroin (Cebra, 1961) on the other hand.

The antigenic specificity relationships described in the present paper demonstrate that synthetic polypeptide antigens, similarly to natural antigens, may possess a rather narrow immune specificity.

SUMMARY

1. Rabbit antisera to several synthetic linear and multichain copolypeptides, containing tyrosine as well as glutamic acid, alanine and/or lysine, were allowed to react with various chemically related and unrelated linear and multichain polypeptides, proteins and polypeptidyl proteins. The extent of reaction was followed by cross-precipitation or by inhibition of the homologous reaction.

2. The synthetic polypeptide antigens investigated contain determinants of well-defined and rather narrow immune specificity. For example, antibodies to p(Tyr,Glu) and to p(Tyr,Glu)--pLys (nomenclature of Sela *et al.* 1962) reacted exclusively with substances containing tyrosine and glutamic

acid. Alanine, when present in the immunogenic molecule, also contributes to specificity.

3. Substances which are not immunogenic, because the area important for immunogenicity is not accessible to the biosynthetic site, may nevertheless cross-react with antibodies formed against substances closely related chemically. Apparently the capacity of a molecule to react with the combining site of an antibody is not parallel with its ability to elicit the formation of antibodies.

4. The antigenic specificity is governed by the area of the molecule most exposed to the surroundings. Thus tyrosine and glutamic acid contribute more to the specificity of p(Tyr,Glu)-pAla--pLys, whereas alanine contributes more to that of pAla-p(Tyr,Glu)--p(Lys,Ala).

5. Antibodies to linear polypeptide antigens gave much better precipitation with linear than with multichain polymers.

6. The efficiency of inhibition of some systems with specificity directed to peptides of tyrosine and glutamic acid by means of copolymers of tyrosine and glutamic acid was strongly dependent on the molar ratio of two amino acids in the copolymer. Some copolymers caused complete inhibition of the homologous reaction of the system p(Tyr,Glu)--p-Lys–anti-p(Tyr,Glu)-pLys. The molar ratios of two such inhibitors to the immunogen at 50% of the total inhibition were 70 and 94.

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Studies on Incorporation of Deuterium into Bacteria

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The mutagenic effect of deuterium oxide has been reported for several strains of bacteria (De Giovanni & Zamenhof, 1959; De Giovanni, 1960, 1961) and for the bacteriophage T4 (Konrad, 1960). With bacteria the growth of several strains was inhibited by media containing deuterium oxide and the degree of inhibition was strain-specific. No adaptation to better growth in media containing deuterium oxide was obtained in any of the strains tested; however, a deuterium-resistant mutant was obtained from one strain. Two strains tested formed enlarged distorted cells when grown for more than eight generations in media containing deuterium oxide. Deuterium induced the occurrence of forward mutants in some strains. Many loci, but not all, showed an up to 54-fold increase in the back-mutation rate over the spontaneous level, indicating some specificity of action by deuterium. Deuterated bases, obtained from a strain grown in a medium containing deuterium oxide, did not induce any phenotypic or genotypic effects when supplied to specific base-requiring strains.

The mechanism of such mutagenic action is not understood; however, it may be related to the extent of deuterium incorporation by bacteria or phage. Differences in the nuclear mass of hydrogen and deuterium may possibly cause disturbances in the synthesis of DNA, leading to permanent changes in its structure and consequently in the genotype it controls.

The purpose of the present study was to investigate the extent of deuterium incorporation in cells of *Escherichia coli* and in nitrogenous bases of their DNA, in an attempt to correlate such incorporation