Relation between Optical Configuration and Immunogenicity of Synthetic Polypeptides

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(*Received* 21 *December* 1964)

1. Three random linear copolymers composed of two or three of the amino acids D-tyrosine, D-glutamic acid, D-alanine and D-lysine, and a branched multichain copolymer with a poly-D-lysine backbone and polymeric side chains of D-tyrosine and D-glutamic acid, were found to be non-antigenic in rabbits, by precipitin and passive cutaneous anaphylaxis, and in guinea pigs, by delayed hypersensitivity tests. The corresponding four copolymers of L-amino acids were shown to be antigenic by all the three criteria. 2. No immunological cross-reactions were observed between the polypeptides composed of D-amino acids and the corresponding L-amino acid copolymers. 3. Similarly, an azobenzenearsonic acid conjugate of poly-D-tyrosine was shown to be non-antigenic in guinea pigs, in contrast with an analogous conjugate of poly-L-tyrosine. Animals sensitized with the conjugate of poly-L-tyrosine did not exhibit delayed skin reactions, when cross-tested with the D-conjugate. 4. A linear polymer composed of D-tyrosine, L-glutamic acid and L-alanine was found to be immunogenic and to cross-react with the corresponding polymer composed exclusively of D-amino acids.

The availability of synthetic polypeptide antigens (Sela & Arnon, 1960*a*; Sela, Fuchs & Arnon, 1962; Gill & Doty, 1960, 1961; Maurer, 1964) allows, through a study of copolymers showing only limited variations in their chemical formulae, information to be obtained about the role of various structural features in their antigenic function. This paper is concerned with the question whether, and to what extent, the different optical isomers of amino acids are capable of contributing to the immunogenicity of a molecule.

Differences between optical isomers of organic compounds may be detected by immunological methods (Landsteiner & van der Scheer, 1929; Avery & Goebel, 1929; Landsteiner, 1945). The distinct serological specificity of antigenic determinants of different optical configurations has also been demonstrated for optical isomers of amino acids. Thus poly-γ-D-glutamic acid was found to be the specific haptenic substance of the capsule of *Bacillus anthracis* (Ivanovics & Bruckner, 1937*a*,*b*,*c*; Ivanovics, 1940), and antibodies with specificity directed towards peptides of D-alanine or of L-alanine were produced in rabbits on immunization with poly-D-alanyl or poly-L-alanyl derivatives of bovine serum albumin (Sage, Deutsch, Fasman & Levine, 1964).

The above studies were concerned with the role of D-amino acids in antigenic specificity, and Sela & Fuchs (1964) have shown that the attachment of peptides of D-tyrosine to gelatin resulted, similarly to the attachment of peptides of L-tyrosine (Sela & Arnon, 1960*b*), in a definite increase in antigenicity as compared with unmodified gelatin. Antibodies to poly-D-tyrosyl gelatin cross-reacted only to a small extent with poly-L-tyrosyl gelatin, and the same situation was observed in the reverse case. Further, the attachment of peptides of D-tyrosine and L-glutamic acid to the non-antigenic multichain poly-DL-alanine was found to convert it into an immunogen (Sela, Fuchs & Givol, 1963; Sela & Fuchs, 1964). The contribution of D-tyrosine to immunogenicity is apparent here, as the attachment of peptides of L-glutamic acid alone does not confer antigenic properties on multichain poly-DL-alanine (Sela *et al.* 1962).

Attempts to elicit an immune response to several linear and multichain synthetic polypeptides composed exclusively of D-amino acids have been largely unsuccessful (Gill, Gould & Doty, 1963; Maurer, 1963; Sela & Fuchs, 1964), although a notable exception to this has been shown (Gill, Kunz, Gould & Doty, 1964). At the same time the corresponding copolymers of L-amino acids were good immunogens, giving positive precipitin reactions with homologous rabbit sera.

Since the antigenicity of synthetic polypeptides can be detected also by the passive cutaneous

anaphylaxis technique (Maurer & Cashman, 1963*a*,*b*; Ben-Efraim, Fuchs & Sela, 1964) and by the delayed hypersensitivity response in guinea pigs (Gill & Doty, 1961; Maurer & Cashman, 1963*a*,*b*; Ben-Efraim, Fuchs & Sela, 1963), we have extended the studies on copolymers of D-amino acids to these immune reactions.

In view of the recent report by Gill *et al.* (1964) that a copolymer of D-tyrosine, D-glutamic acid and D-lysine was found to be antigenic in rabbits, when tested by precipitin reaction, we examined a polymer of similar composition for its possible antigenicity in guinea pigs and also in rabbits.

Azobenzenearsonate–poly-L-tyrosine was found to be non-antigenic in rabbits (Sela & Haurowitz, 1958), but a similar conjugate was found to elicit a delayed response in guinea pigs (Leskowitz, 1963). In view of this latter finding, we examined the response of guinea pigs towards azobenzenearsonate–poly-D-tyrosine.

MATERIALS

The synthesis and physicochemical characterization of the polypeptides listed in Table 1 were accomplished according to the methods described by Sela *et al.* (1962). Their physical, chemical and immunological properties, with the exceptions of materials nos. 251 and 252, have been described in detail by Sela & Fuchs (1964). The nomenclature used below for the synthetic polypeptides is that adopted by Sela *et al.* (1962).

The linear polymers p(D-Tyr,D-Glu,D-Lys) (no. 251) and p(L-Tyr,L-Glu,L-Lys) (no. 252) were prepared according to the method described by Sela *et al.* (1962). The original molar proportions of the *N*-carboxyanhydrides of tyrosine, γ-benzyl glutamate and ϵ-benzyloxycarbonyl-lysine were 6:55:39 in both cases. The physicochemical data for these two polymers are shown in Table 1.

Table 1. *Composition and molecular weights of* L- *and* D-*amino acid polymers*

No. and designation of sample	Molar proportions of amino acid residues in the copolymer	Average mol.wt.
102, p(L-Tyr,L-Glu)*	1:1	12400†
237, p(D-Tyr,D-Glu)	1:1·2	13400†
42, p(L-Tyr,L-Glu,L-Ala)*	1:5·4:3·9	4100‡
236, p(D-Tyr,D-Glu,D-Ala)	1:5·8:5·1	33800‡
213, p(D-Tyr,L-Glu,L-Ala)	1:6·8:3·7	21400‡
252, p(L-Tyr,L-Glu,L-Lys)	1:9·5:6·4	61000‡
251, p(D-Tyr,D-Glu,D-Lys)	1:10·4:6	44000‡
249, p(L-Tyr,L-Glu)--p-L-Lys	1:7·6:4	149000§
248, p(D-Tyr,D-Glu)--p-D-Lys	1:8·3:3·6	169000§

* Sela *et al.* (1962).

† From amino nitrogen determinations.

‡ From sedimentation and diffusion measurements.

§ From amino acid analysis and the molecular weight of the polylysine backbone.

The azobenzenearsonic acid derivatives of polytyrosine (cf. Sela & Haurowitz, 1958; Leskowitz, 1963) were prepared and characterized as follows.

Azobenzenearsonate–poly-L-tyrosine. Poly-L-tyrosine was prepared according to Katchalski & Sela (1953), and characterized by sedimentation and diffusion in dimethylformamide solution. An average molecular weight of 137000 (degree of polymerization, $n = 840$) was calculated from the sedimentation coefficient, $S_{20,DMF} = 3{\cdot}35$ S, diffusion coefficient, $D_{20,DMF} = 1{\cdot}8 \times 10^{-7}$ cm.^{2}/sec., and a partial specific volume of tyrosine residue, $\bar{v} = 0{\cdot}71$ ml./g. (Cohn & Edsall, 1943).

A solution of 50mg. of poly-L-tyrosine in 5ml. of N-sodium hydroxide and 5ml. of N-sodium carbonate was treated at 2° with a diazonium salt prepared from 90mg. of *p*-arsanilic acid in 7ml. of 0·2N-hydrochloric acid and 30mg. of sodium nitrite dissolved in 1ml. of water. The mixture was shaken in the cold overnight; it was then dialysed for 3 days against distilled water and freeze-dried. The coloured product was shown to be homogeneous by agar-gel electrophoresis in 0·025M-barbital buffer, pH 8·2, at 300v for 1hr., where it moved to the anode as a discrete spot. It was analysed for its *p*-azobenzenearsonic acid content by the colorimetric analysis of its solution in 0·1N-sodium hydroxide at 460 and 500mμ, by using the molar extinction coefficients for mono-(*p*-azobenzenearsonic acid)-*N*-chloroacetyltyrosine (Tabachnick & Sobotka, 1960) as well as by the determination of arsenic in a sample, with acid digestion followed by iodometric titration (Evers & Smith, 1955). The results showed the presence of one *p*-azobenzenearsonic acid group per 18 tyrosine residues, or 46 substituents per molecule of polymer.

Azobenzenearsonate–poly-D-tyrosine. This material was prepared from poly-D-tyrosine with an average molecular weight of 102000, calculated from the sedimentation coefficient, $S_{20,DMF} = 2{\cdot}90$ S, diffusion coefficient, $D_{20,DMF} = 2{\cdot}1 \times 10^{-7}$ cm.^{2}/sec. and partial specific volume, $\bar{v} = 0{\cdot}71$ ml./g. (degree of polymerization, $n = 626$). The method of preparation was analogous to that described above for poly-L-tyrosine. The product was found to contain one *p*-azobenzenearsonic group per 19 tyrosine residues, or 33 substituents per molecule of polymer.

METHODS

Immunization procedures. Each D-amino acid polymer was injected into the thighs of hind legs of eight rabbits, after emulsifying equal parts of 2·5% polymer solution in aq. 0·9% sodium chloride and complete Freund adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.), four times at 10-day intervals, 1·0ml. of the emulsion being used per injection. After that, each animal was injected intravenously for 4 consecutive days with 0·5ml. of 0·2% polymer solution in aq. 0·9% sodium chloride. The animals were bled before each injection and over the period of 3 months after the last injection.

The preparation of the rabbit antisera to 42, p(L-Tyr,L-Glu,L-Ala), and to 102, p(L-Tyr,L-Glu), was described by Sela *et al.* (1962). Rabbit antisera to other L-amino acid copolymers were obtained by the same procedure.

All the sera were treated with thiomersal (final concn. 0·01%) and frozen until the time of their examination.

The rabbits used throughout the experiments were non-albinos of both sexes, bred at the Weizmann Institute, with an average weight of 2·5kg.

With guinea pigs, each animal received a single injection of 0·2ml. of emulsion containing equal parts of the polymer solution in aq. 0·9% sodium chloride and complete Freund adjuvant, distributed between the footpads of the hind legs. The sensitizing dose was 1mg. for the D-amino acid polymers including p(D-Tyr,L-Glu,L-Ala) and 0·1mg. for the L-amino acid polymers. The animals were tested by intradermal injections with solutions of 0·05mg./0·1ml. of a given polymer in aq. 0·9% sodium chloride on the tenth and eighteenth days after the sensitizing injection. The skin reactions were read and recorded after 2hr. and then after 24hr. The guinea pits were bled after each skin-test and the sera were frozen until the time of their examination.

The guinea pigs used were randomly bred, Hartley strain female albinos, weighing 300–400g. They were purchased from Camm Research Institute Inc., Wayne, N.J., U.S.A.

Quantitative precipitin tests. These were carried out with rabbit sera according to the procedure described by Sela *et al.* (1962), by using the homologous polypeptides, as well as polypeptides of the opposite optical configuration, as test antigens.

Passive cutaneous anaphylaxis. The procedure used in this test was based on that described by Ovary (1958). Sera from animals injected with D-amino acid polymers were tested in undiluted form (0·1ml. portions), and sera from animals immunized with L-amino acid polymers were tested with 0·2ml. portions of 1:2 and 1:20 dilutions. The polypeptides were injected intracardially in doses of 5mg./ml., mixed with 1ml. of 1% solution of Evans Blue, 18hr. after the intradermal injections of sera (cf. Ben-Efraim *et al.* 1964). The reactions were recorded as positive only if their average diameter was larger than 5mm. Each test was performed on two or three animals.

Spectrophotometric measurements. These were made on a Zeiss model PMQII spectrophotometer, at approx. 25°, with quartz cells of 1cm. light-path.

Sedimentation analysis. This was carried out in a Spinco model E ultracentrifuge, at 19–24°, with the schlieren optical system. The samples were sedimented at 59 780rev./min. The readings were corrected to 20°.

Diffusion measurements. These were performed in the same Spinco model E ultracentrifuge (Daniel & Katchalski, 1962). The boundary between the solvent and the solution was obtained with a synthetic-boundary cell and operating at a low gear (8766rev./min.). At this speed the sedimentation of the polymers investigated was practically negligible.

Amino acid analysis. The amino acid contents of polypeptides were determined by quantitative analysis with the Beckman–Spinco model 120B automatic amino acid analyser (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). The samples to be analysed were first subjected to hydrolysis with 6N-hydrochloric acid in sealed tubes at 110° for 24hr.

RESULTS

None of the polymers composed exclusively of D-amino acids was found to be antigenic in the rabbits or in the guinea pigs, under the described conditions of immunization and testing. As seen in Table 2, the D-amino acid polymers did not elicit antibody formation in rabbits as tested by precipitin or passive cutaneous anaphylaxis, nor did they provoke an immune response in guinea pigs, as shown by the absence of delayed hypersensitivity and passive cutaneous anaphylaxis reactions. This was true also for sensitization of guinea pigs with 0·1mg. doses of the D-amino acid polymers.

On the other hand, all of the corresponding L-amino acid polymers as well as p(D-Tyr,L-Glu,L-Ala) were shown to be antigenic in both species of animals. The precipitin reactions recorded in Table 2 as positive corresponded to the amounts of antibody precipitated at the equivalence point within the range 400–2000μg./ml. In all cases, at least 70% of the rabbits immunized with L-amino acid polymers were found to contain precipitating antibodies directed against the immunizing antigen. All of the precipitin-positive sera were shown also to give positive passive cutaneous anaphylaxis. Three rabbits, which had been injected with p(D-Tyr,D-Glu,D-Lys) and shown to be unresponsive, were subsequently immunized with p(L-Tyr,L-Glu,L-Lys); two produced precipitating antibodies.

None of the test antigens gave any precipitate when added to pre-immunization sera.

All of the polymers found to be antigenic in the rabbits were also capable of eliciting an immune response in the guinea pigs. The results of skin tests read after 24hr. and recorded in Table 2 are those obtained 18 days after sensitization, except the data for p(D-Tyr,L-Glu,L-Ala), recorded 10 days after sensitization. All of the L-amino acid polymers tested induced delayed hypersensitivity in the guinea pigs. Some of the sensitized animals were also found to contain circulating antibodies detectable by the passive cutaneous anaphylaxis reactions. Out of eight guinea pigs giving delayed reactions with p(L-Tyr,L-Glu,L-Lys), four had been previously injected with p(D-Tyr,D-Glu,D-Lys) and found unresponsive.

No cross-reactions, in precipitin or passive cutaneous anaphylaxis tests, were observed between the sera from rabbits immunized with L-amino acid polymers and the corresponding D-amino acid polymers. Similarly, among the guinea pigs giving positive delayed skin reactions with the L-amino acid polymers, none cross-reacted in this way with the corresponding D-amino acid polymers. On the other hand, such cross-reactions were observed in guinea pigs between the copolymer of D-tyrosine, L-glutamic acid and L-alanine, and the corresponding copolymers composed exclusively of D- or of L-amino acids. Pooled rabbit anti-p(D-Tyr,L-Glu,L-Ala) sera cross-reacted with p(D-Tyr,D-Glu,D-Ala) by passive cutaneous anaphylaxis but not by the precipitin test.

Table 2. *Antigenicity of* L- *and* D-*amino acid polymers*

PCA, Passive cutaneous anaphylaxis; ND, not determined.

Immunizing antigen	Test antigen	Antibody response in the rabbit		Immune response in the guinea pig		
				Positive delayed skin reactions		No. of animals with PCA-positive sera
		Precipitin	PCA	No. of animals	Average diameter (mm.)	
102, p(L-Tyr,L-Glu)	102, p(L-Tyr,L-Glu)	+	+	5/5	14	0/5
	237, p(D-Tyr,D-Glu)	−	−	0/5	0	ND
237, p(D-Tyr,D-Glu)	237, p(D-Tyr,D-Glu)	−	−	0/6	0	0/6
	102, p(L-Tyr,L-Glu)	−	−	0/6	0	ND
42, p(L-Tyr,L-Glu,L-Ala)	42, p(L-Tyr,L-Glu,L-Ala)	+	+	5/5	11	0/5
	236, p(D-Tyr,D-Glu,D-Ala)	−	−	0/6	0	0/6
236, p(D-Tyr,D-Glu,D-Ala)	236, p(D-Tyr,D-Glu,D-Ala)	−	−	0/5	0	ND
	42, p(L-Tyr,L-Glu,L-Ala)	−	−	0/6	0	ND
213, p(D-Tyr,L-Glu,L-Ala)	213, p(D-Tyr,L-Glu,L-Ala)	+	+	3/4	14	1/4
	236, p(D-Tyr,D-Glu,D-Ala)	−	+	3/4	7	ND
	42, p(L-Tyr,L-Glu,L-Ala)	+	+	3/4	10	ND
252, p(L-Tyr,L-Glu,L-Lys)	252, p(L-Tyr,L-Glu,L-Lys)	+	+	8/9	13	3/9
	251, p(D-Tyr,D-Glu,D-Lys)	−	−	0/9	0	0/9
251, p(D-Tyr,D-Glu,D-Lys)	251, p(D-Tyr,D-Glu,D-Lys)	−	−	0/10	0	0/10
	252, p(L-Tyr,L-Glu,L-Lys)	−	−	0/10	0	ND
249, p(L-Tyr,L-Glu)--p-L-Lys	249, p(L-Tyr,L-Glu)--p-L-Lys	+	+	3/3	15	ND
	248, p(D-Tyr,D-Glu)--p-D-Lys	−	−	0/3	0	ND
248, p(D-Tyr,D-Glu)--p-D-Lys	348, p(D-Tyr,D-Glu)--p-D-Lys	−	−	0/3	0	ND
	249, p(L-Tyr,L-Glu)--p-L-Lys	−	−	0/3	0	ND
Azobenzenearsonate–p-L-Tyr	Azobenzenearsonate–p-L-Tyr	ND	ND	3/4	13	0/4
	Azobenzenearsonate–p-D-Tyr	ND	ND	0/4	0	ND
Azobenzenearsonate–p-D-Tyr	Azobenzenearsonate–p-D-Tyr	ND	ND	0/4	0	0/4
	Azobenzenearsonate–p-L-Tyr	ND	ND	0/4	0	ND

The attachment of azobenzenearsonate groups converted poly-L-tyrosine, a homopolymer which thus far has been shown incapable of eliciting an immune response (R. Arnon & M. Sela, unpublished work), into an antigen (see also Leskowitz, 1963). A similar modification of poly-D-tyrosine did not make it antigenic. Furthermore, azobenzenearsonate–poly-D-tyrosine did not give delayed cross-reactions in guinea pigs sensitized to azobenzenearsonate–poly-L-tyrosine, even though such animals had been observed to cross-react with several azobenzenearsonate–protein conjugates (Leskowitz, 1963; F. Borek, Y. Stupp & M. Sela, unpublished work).

DISCUSSION

The results presented in this paper show that a series of synthetic polypeptides composed exclusively of L-amino acids, as well as a copolymer of D-tyrosine, L-glutamic acid and L-alanine, were immunogenic in rabbits and elicited delayed hypersensitivity in guinea pigs. On the other hand, linear and multichain polypeptides composed exclusively of D-amino acids failed to elicit an immune response in rabbits and in guinea pigs, as shown by negative precipitin, passive cutaneous anaphylaxis and delayed hypersensitivity reactions. Similarly, a conjugate of *p*-azobenzenearsonic acid with poly-D-tyrosine was unable to sensitize guinea pigs, in contradistinction to the L-conjugate.

Thus, even though attachment of D-tyrosine peptides may enhance or confer immunogenicity on poorly antigenic or non-antigenic macromolecules containing L-amino acids (Sela *et al.* 1963; Sela & Fuchs, 1964), polymers devoid of L-amino acids do not seem to be able to elicit an immune response. This conclusion agrees, with one exception (Gill *et al.* 1964), with the results obtained in similar experiments on D-amino acid copolymers in other Laboratories and summarized in Table 3.

An attempted immunization of a rabbit or of a guinea pig with a D-amino acid polymer had no effect on the subsequent immune response of the animal to the corresponding L-amino acid polymer (see also Levine, 1964). Thus the D-polypeptides do

Table 3. *Immunological properties of polymers composed exclusively of* D-*amino acids*

R, Rabbit; GP, guinea pig.

Polymer	Amino acid proportions	Mol. wt.	Species studied	Antigenic*	Reference
p(D-Glu,D-Ala)	6:4	47000	R	No	Maurer (1963)
p(D-Glu,D-Lys)	5·7:4·3	43000	R	No	Gill *et al.* (1963)
p(D-Tyr,D-Glu)	1:1·2	13400	R and GP	No	This paper
p(D-Tyr,D-Glu,D-Ala)	1:6:3	33000	R	No	Maurer (1963)
p(D-Tyr,D-Glu,D-Ala)	1:5·8:5·1	33800	R and GP	No	This paper
p(D-Tyr,D-Glu,D-Ala)	1:5·6:4·4	19700	R	No	Sela & Fuchs (1964)
p(D-Tyr,D-Glu,D-Lys)	1:9·2:6·5	93000	R	Yes	Gill *et al.* (1964)
p(D-Tyr,D-Glu,D-Lys)	1:10·4:6	44000	R and GP	No	This paper
p(D-Tyr,D-Glu)--p-D-Lys	0·28:2·3:1	169000	R and GP	No	This paper
Azobenzenearsonate–p-D-Tyr	—	123400	GP	No	This paper
Azobenzenearsonate–p(D-Tyr,D-Glu,D-Ala)	1:6:3	34400	GP	No	Benacerraf, Ojeda & Maurer (1963)
Benzylpenicilloyl-p-D-Lys	—	36200	GP	No	Parker & Thiel (1963)
Benzylpenicilloyl-p-D-Lys	—	25100	GP (strain 2)	No	Levine (1964)

* All the L-amino acid polymers corresponding to the D-amino acid polymers listed in this Table were found to be antigenic.

not seem to interfere with the steps leading to a normal immune response towards the L-antigens.

None of the D-polypeptides investigated cross-reacted by precipitin test or by passive cutaneous anaphylaxis with antisera directed against the corresponding L-polypeptides, nor were guinea pigs sensitized with the L-polymers reactive towards their D-analogues. This is consistent with the findings of Gill *et al.* (1963, 1964) that p(D-Glu,D-Lys) and p(D-Tyr,D-Glu,D-Lys) did not cross-react with rabbit antisera directed against the respective L-polypeptides, when tested by the precipitin method, nor did the D-polypeptides inhibit the homologous reaction with the corresponding L-polypeptides. The authors concluded that the antibody-recognition area on the polypeptide molecule is not limited to the amino acid side groups, but rather extends to a section of the peptide chain. A stereospecific reaction was also observed in D-alanine peptides linked to protein (Sage *et al.* 1964). On the other hand, Maurer (1963) found that although p(D-Glu,D-Ala) and p(D-Tyr,D-Glu,D-Ala) did not precipitate or fix complement with antibodies produced against the L-polymers and did not inhibit the homologous precipitin reaction, they did cross-react in the passive cutaneous anaphylaxis reaction with the respective anti-L-polymer sera. No explanation seems to be available at present for the discrepancy between the negative passive cutaneous anaphylaxis cross-reactions reported in this paper and the positive results of Maurer (1963).

In contrast with the lack of reaction between the anti-L-polymer sera and the D-polymers, the antisera against p(D-Tyr,L-Glu,L-Ala), a polypeptide containing amino acids of both optical configurations, cross-reacted with p(D-Tyr,D-Glu,D-Ala) by passive cutaneous anaphylaxis, though not by the precipitin test. This is consistent with the findings on the role of D-tyrosine in the immunological specificity of poly-D-tyrosyl gelatin and of p(D-Tyr,L-Glu)-p-DL-Ala--p-L-Lys (Sela & Fuchs, 1964).

Bancerraf *et al.* (1963) reported that azobenzenearsonate–p(D-Tyr,D-Glu,D-Ala) cross-reacted by passive cutaneous anaphylaxis with the anti-azobenzenearsonate sera obtained from guinea pigs immunized with the azobenzenearsonate–L-copolymer. Cross-reactions were also obtained by skin tests with the azobenzenearsonate–D-copolymer of guinea pigs sensitized to the azobenzenearsonate-L-copolymer. In contrast, as shown in Table 3, no cross-reactions with azobenzenearsonate–p-D-Tyr in guinea pigs sensitized to azobenzenearsonate–p-L-Tyr were observed in this study. This may be because azobenzenearsonate–p-L-Tyr is a considerably weaker antigen than azobenzenearsonate–p(L-Tyr,L-Glu,L-Ala), as shown by the respective magnitudes of homologous delayed skin reactions.

The only report thus far made of an antigenic D-amino acid copolymer is that of Gill *et al.* (1964). After having found p(D-Glu,D-Lys) (5·7:4·3) to be non-antigenic in the rabbit, these authors investigated the antigenicity of p(D-Tyr,D-Glu,D-Lys) (1:9·2:6·5), since they knew that, in the L-polymer series, introduction of tyrosine increases the capacity of the given polymer to elicit an antibody response in the rabbit (Gill & Doty, 1961; Sela *et al.* 1962). This D-polymer did elicit antibody formation in rabbits, though the amount of antibody found by the precipitin reaction was only one-quarter to one-third of the amount formed against the corre-

sponding L-polymer. Our attempts to elicit an immune response in rabbits and guinea pigs by the use of a D-polypeptide with a composition practically identical with that employed by Gill *et al.* (1964), though of a lower molecular weight (cf. Table 3), and by applying a similar immunization schedule, were not successful. It is not clear whether the discrepancy between these results is due to the difference in the molecular weights of the polymers involved, to a genetic difference in the animals used in the two Laboratories or to some other unspecified reasons.

Regardless of whether no polypeptides composed exclusively of D-amino acids may be immunogenic in any species, or whether (as suggested by the report of Gill *et al.* 1964) D-polypeptides of certain compositions may elicit some antibody, there is no doubt that the difference in immunogenicity between polypeptides containing amino acids of opposite optical configurations is a striking one. Several explanations of this difference have been suggested (Sela, 1965).

We thank Mrs Sara Ehrlich-Rogozinski for the analyses of arsenic, and acknowledge the competent technical assistance of Mr Israel Jacobson and Miss Noa Novik. This research has been supported by the U.S. Air Force under Grant 64-22, European Office of Aerospace Research. F. B. was a recipient of a U.S. Public Health Service Fellowship (no. 1-F3-AI-19910-01) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

Avery, O. T. & Goebel, W. F. (1929). *J. exp. Med.* **50**, 533.
Benacerraf, B., Ojeda, A. & Maurer, P. H. (1963). *J. exp. Med.* **118**, 945.
Ben-Efraim, S., Fuchs, S. & Sela, M. (1963). *Science*, **139**, 1222.
Ben-Efraim, S., Fuchs, S. & Sela, M. (1964). *Immunology*, **7**, 142.
Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides*, p. 375. New York: Reinhold Publishing Corp.
Daniel, E. & Katchalski, E. (1962). In *Polyamino Acids, Polypeptides and Proteins*, p. 183. Ed. by Stahmann, M. A. Madison: University of Wisconsin Press.
Evers, N. & Smith, W. (1955). *The Analysis of Drugs and Chemicals*, pp. 87–88. London: C. Griffin and Co. Ltd.
Gill, T. J., III & Doty, P. (1960). *J. molec. Biol.* **2**, 65.
Gill, T. J., III & Doty, P. (1961). *J. biol. Chem.* **236**, 2677.
Gill, T. J., III, Gould, H. J. & Doty, P. (1963). *Nature, Lond.*, **197**, 746.
Gill, T. J., III, Kunz, H. W., Gould, H. J. & Doty, P. (1964). *J. biol. Chem.* **239**, 1107.
Ivanovics, G. (1940). *Z. ImmunForsch.* **97**, 402.
Ivanovics, G. & Bruckner, V. (1937*a*). *Naturwissenschaften*, **25**, 250.
Ivanovics, G. & Bruckner, V. (1937*b*). *Z. ImmunForsch.* **90**, 304.
Ivanovics, G. & Bruckner, V. (1937*c*). *Z. ImmunForsch.* **91**, 175.
Katchalski, E. & Sela, M. (1953). *J. Amer. chem. Soc.* **75**, 5284.
Landsteiner, K. (1945). *The Specificity of Serological Reactions*, 2nd revised ed. Cambridge, Mass.: Harvard University Press.
Landsteiner, K. & van der Scheer, J. (1929). *J. exp. Med.* **50**, 407.
Leskowitz, S. (1963). *J. exp. Med.* **117**, 909.
Levine, B. B. (1964). *Nature, Lond.*, **202**, 1008.
Maurer, P. H. (1963). *Proc. Soc. exp. Biol., N.Y.*, **113**, 553.
Maurer, P. H. (1964). *Progr. Allergy*, **8**, 1.
Maurer, P. H. & Cashman, T. (1963*a*). *J. Immunol.* **90**, 393.
Maurer, P. H. & Cashman, T. (1963*b*). *J. Immunol.* **90**, 493.
Ovary, Z. (1958). *Progr. Allergy*, **5**, 1.
Parker, C. W. & Thiel, J. A. (1963). *J. Lab. clin. Med.* (*Abstr.*), **62**, 998.
Sage, H., Deutsch, G. F., Fasman, G. & Levine, L. (1964). *Immunochemistry*, **1**, 133.
Sela, M. (1965). *Advanc. Immunol.* **5**, (in the Press).
Sela, M. & Arnon, R. (1960a). *Biochim. biophys. Acta*, **40**, 382.
Sela, M. & Arnon, R. (1960*b*). *Biochem. J.* **75**, 91.
Sela, M. & Fuchs, S. (1964). *Proc. Symp. Molecular and Cellular Basis of Antibody Formation, Prague*, p. 43. Prague: Czechoslovak Academy of Sciences.
Sela, M., Fuchs, S. & Arnon, R. (1962). *Biochem. J.* **85**, 223.
Sela, M., Fuchs, S. & Givol, D. (1963). *Abstr. Pap. Amer. chem. Soc., 143rd Meet., Cincinnati*, p. 9a.
Sela, M. & Haurowitz, F. (1958). *Experientia*, **14**, 91.
Tabachnick, M. & Sobotka, H. (1960). *J. biol. Chem.* **235**, 1051.