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Preferential Formation of Antibodies Specific toward D-Amino Acid Residues upon Immunization with Poly-DL-peptidyl Proteins*

Israel Schechter and Michael Sela

ABSTRACT: Precipitating antibodies reacting with poly-DL-alanyl determinants were produced in rabbits upon immunization with poly-DL-alanyl proteins. The specificity of these antibodies was directed mainly toward the D-alanine sequences in the poly-DL-alanyl determinants, as concluded from cross-precipitation reactions, absorption experiments, immunodiffusion tests, and inhibition studies with alanine peptides. The preferential immune response to determinants composed of D-amino acids was also observed when poly-DL-phenylalanyl and poly-DL-tyrosyl proteins were used for immunization.

The above findings may be interpreted as antigenic competition between sequences composed of L-, D-, or DL-amino acids, with the D sequences being the most efficient.

Polypeptidyl proteins are proteins to which peptide chains are attached. From the point of view of their immunological properties, the attached peptides may be considered haptens. Polypeptidyl protein antigens have been used in immunological studies concerned with immunogenicity, antigenic specificity, and immunological tolerance (Sela, 1966).

Investigations of antibodies to poly-L-alanyl and poly-D-alanyl determinants were reported recently (Sage *et al.*, 1964; Schechter and Sela, 1965a; Schechter *et al.*, 1966). The extent of formation of antibodies with specificity directed toward either determinant was of the same order of magnitude. The antibodies formed were strictly stereospecific as expected. When immunoglobulin G preparations devoid of proteolytic activity (Schechter *et al.*, 1966) were used, the size of the specific combining region of the antibodies was found such as to accommodate a maximum of three to four alanine residues, and the region of the antigenic determinant farthest removed from the protein carrier was of paramount importance in determining the specificity of the antibodies formed, as concluded from inhibition experiments with alanine peptides (Schechter *et al.*, 1966).

In view of the above results it was of interest to elucidate the specificity of the anti-polyalanyl antibodies formed upon immunization with poly-DL-alanyl pro-

* From the Section of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel. *Received October 25, 1966.* Supported in part by Research Grant AI-04715 from the National Institutes of Health, U. S. Public Health Service. Part of this work has appeared in the form of a preliminary note (Schechter and Sela, 1965b).

teins. The present study shows that immunization of rabbits and goats with poly-DL-alanyl proteins leads to the formation of antibodies with specificity directed predominantly to peptide sequences of D-alanine. This seems a general phenomenon, as poly-DL-phenylalanyl and poly-DL-tyrosyl proteins have elicited in rabbits antipolypeptide antibodies with specificity directed mainly to D-amino acid sequences. This may serve as an example of the phenomenon of antigenic competition between sequences composed of L-, D-, and DL-amino acid residues, the D sequences being the most efficient.

Materials and Methods

Rabbit serum albumin[1] (fraction V, powder) was purchased from Mann Research Laboratories, New York, N. Y. Human serum albumin (fraction V, powder) was obtained from Plasma Fractionation Institute of Israel, Jaffa-TelAviv. Bovine serum albumin (fraction V, powder) was supplied by Armour Pharmaceutical Co., Eastbourne, England. Ribonuclease A, (five times recrystallized, lot 63B-1010), and leucine aminopeptidase, (type II, lot 14B-0300), were purchased from Sigma, St. Louis, Mo. Noble agar and complete Freund adjuvant were obtained from Difco laboratories, Detroit. *N*-Carboxyanhydrides of alanine, phenylalanine, and tyrosine were prepared by reacting phosgene with the respective amino acids in dioxane, essentially according to the procedures described previously (Katchalski and Sela, 1958), followed by recrystallization from ethyl acetate–petroleum ether (bp 30–60°) at −20°. The alanine peptides used for inhibition studies were synthesized and characterized as described previously (Schechter and Berger, 1966a). All other chemicals used were of analytical reagent grade.

Polypeptidyl proteins used in this study are given in Table I. They were prepared by reacting the protein with the appropriate *N*-carboxyamino acid anhydride in a water–dioxane mixture according to published procedures (Schechter *et al.*, 1964). The amount of amino acid attached to a protein was calculated from the amino acid analysis of hydrolysates before and after peptidylation. The number of peptide chains per protein molecule was obtained by determining the lysine content after desamination of the derivative with nitrous acid (Anfinsen *et al.*, 1962). In the case of polypeptidyl-RNase derivatives the α-amino-terminal group, which serves as an initiator for polymerization similarly to the ϵ-amino groups, was taken into account in calculating the number of peptide chains attached. Differences in the number of chains and in the average size of chains (see Table I) are probably due to contamination of the *N*-carboxyamino acid anhydride preparations with polymer formed upon storage.

Rabbit immunoglobulin G (IgG), prepared by chromatography on DEAE-cellulose (Levy and Sober, 1960), was found to be devoid of proteolytic activity (Schechter *et al.*, 1966).

Amino Acid Analysis. The protein samples were subjected to hydrolysis with 6 N hydrochloric acid in sealed tubes at 110° for 24 hr. The amino acids were then determined quantitatively (Spackman *et al.*, 1958) with the Beckman–Spinco automatic amino acid analyzer Model 120B.

Activity of leucine aminopeptidase on polypeptidyl proteins was determined in 0.05 M sodium-barbital buffer, pH 8.0, and 0.005 M $MnSO_4$. The reaction mixture contained in 1 ml of 80 μg of LAP and 50 mg of polypeptidyl protein. After 5 hr at 37° a sample of 10 μl was loaded on a Whatman No. 1 paper sheet which was chromatographed with *n*-butyl alcohol–acetic acid–water (25:6:25), upper layer) for 24 hr. The spots were detected with ninhydrin.

Immunization Procedure. Randomly bred rabbits (2.5–3.5 kg) of both sexes were used. The antigens were injected (15 mg/injection) intramuscularly in complete Freund adjuvant as described (Schechter *et al.*, 1966). Three injections were given at fortnightly intervals and some rabbits received an additional intravenous injection of 10 mg of antigen, dissolved in 1 ml of 0.9% sodium chloride. Bleeding of the animals was started 2 weeks after the third intramuscular injection.

Quantitative Precipitin Studies. To a constant volume of antiserum or of IgG solution (0.4–1.0 ml) increasing amounts of the precipitant dissolved in 0.9% sodium chloride were added and the final volume was brought to 1.7 ml with 0.9% sodium chloride. The reaction mixture was kept at 37° for 45 min and then at 5° for 2 days. Precipitates formed were separated by centrifugation, washed twice with 2.0 ml of 0.9% sodium chloride, dissolved in 1.1 ml of 0.1 N sodium hydroxide, and the absorbance at 280 mμ was determined.

Absorption experiments were performed by reacting antiserum with one precipitant, and the clear supernatant with the second precipitant. The amount of precipitant added was calculated from the regular precipitin curve to yield maximal precipitation. Each precipitant was reacted with the antiserum or supernatant as described above for quantitative precipitin technique. All absorption experiments were carried out in duplicate.

Quantitative inhibition with alanine peptides was determined by measuring the absorbance of the precipitate (in 0.1 N NaOH) formed in a mixture which contained IgG preparation, peptide, and precipitant. Constant amounts of IgG and precipitant were used. These were calculated from the optimal zone of the regular precipitin curve to yield a precipitate which in 0.1 N NaOH solution will have an absorbance at 280 mμ in the range of 0.4–0.7. The reagents were reacted as follows. Varying amounts of peptide dissolved in 0.9% sodium chloride were added to a constant volume of IgG solution. The volume was adjusted to 1.5 ml with 0.9% sodium chloride. The mixture was kept at 37° for 45 min. A constant amount of precipitant in 0.2 ml of 0.9% sodium chloride was

[1] Abbreviations used: RSA, rabbit serum albumin; HSA, human serum albumin; BSA, bovine serum albumin; LAP, leucine aminopeptidase; IgG, immunoglobulin G; RGG, rabbit IgG; D_4, tetrapeptide H·Ala-Ala-Ala-Ala·OH·(4D).

TABLE I: Characterization of Polypeptidyl Proteins.

Designation and No. of Derivative	*N*-Carboxyamino Acid Anhydride (g/g of protein)	Moles of Amino Acid per Mole of Protein	Moles of Amino Acid per Mole of Derivative	Moles of Peptide Chains/ Mole of Derivative	Av No. of Amino Acid Residues/ Chain	Sedimentation Coefficient, S (%)[a]
p-DL-Ala-RSA (560)	1 5	54	341	36	8.0	4.0
p-D-Ala-RSA (551)	0.8	54	225	20	8.5	4.1
p-L-Ala-RSA (569)	0.8	54	276	26	8.5	4.0
p-DL-Ala-BSA (530)	2.7	45	308	28	9.4	3.9
p-DL-Ala-HSA (524)	0.5	63	183	31	3.9	3.6
p-DL-Ala-HSA (592_s)	2.0	63	438	35	11	3.7
p-DL-Ala-HSA (543)	6.0	63	1530	15	98	4.6
p-DL-Ala-RNase (540)	1.5	12	94	8.2	10	1.6
p-DL-Ala-RNase (596)	1.5	12	130	9.0	13	1.7
p-DL-Ala-RNase (522)	3.0	12	189	8.3	21	1.7
p-DL-Ala-RNase (541)	4.5	12	175	7.1	23	1.6
p-DL-Ala-RNase (542)	6.0	12	195	5.2	35	1.7
p-D-Ala-RNase (553)	0.8	12	59	7.8	6.9	1.6
p-L-Ala-RNase (565)	0.8	12	76	9.1	7.9	1.6
p-DL-Ala-RGG (8)	5.0	78	931	43	20	
p-DL-Phe-RSA (534)	0.3	23	80	18	3.1	4.2 (50), 6.4 (50)
p-DL-Phe-RSA (606)	0.3	23	88	23	2.8	3.9 (35), 7.0 (65)
p-D-Phe-RSA (586)	0.3	23	81	24	2.4	4.6 (30), 6.4 (70)
p-L-Phe-RSA (585)	0.3	23	75	24	2.1	4.3 (45), 6.6 (55)
p-DL-Phe-BSA (535)	0.3	26	86	25	2.4	3.9 (70), 10 (30)
p-DL-Phe-HSA (590)	0.3	32	100	28	2.4	3.9 (50), 6.3 (50)
p-DL-Phe-HSA (605)	0.3	32	101	27	2.5	3.8 (50), 9.9 (50)
p-D-Phe-HSA (589)	0.3	32	98	26	2.5	4.0 (65), 6.7 (35)
p-L-Phe-HSA (588)	0.3	32	92	26	2.3	3.9 (70), 6.5 (30)
p-DL-Phe-RNase (593)	0.3	3	18	7.0	2.1	1.8 (70), 8.2 (30)
p-D-Phe-RNase (592)	0.3	3	18	5.4	2.8	1.8 (70), 8.9 (30)
p-L-Phe-RNase (591)	0.3	3	17	6.9	2.0	1.8 (70), 8.4 (30)
p-DL-Tyr-RSA (581)	0.3	23	69	20	2.3	4.1 (90), 6.2 (10)
p-D-Tyr-RSA (580)	0.3	23	86	20	3.1	4.2 (60), 6.5 (40)
p-L-Tyr-RSA (545)	0.3	23	85	21	3.0	4.2 (90), 6.2 (10)
p-DL-Tyr-RNase (584)	0.3	6	20	4.5	3.1	1.8
p-D-Tyr-RNase (583)	0.3	6	24	6.0	3.0	1.8
p-L-Tyr-RNase (582)	0.3	6	22	5.8	2.8	1.9

[a] Measured in a Spinco Model E ultracentrifuge on 1% solution at 20°. Polyalanyl conjugates were dissolved in 0.1 M phosphate buffer pH 7.0; polyphenylalanyl and polytyrosyl conjugates were dissolved in 0.1 M phosphate buffer, pH 7.8. The samples were sedimented at 59,780 rpm. When two peaks were observed the sedimentation coefficient of each was measured and the relative areas are enclosed in parentheses. p = poly.

added, and the reaction mixture was kept for an additional 45 min at 37° and then at 5° for 2 days. The precipitates formed were treated as described above for quantitative precipitin technique. All inhibition experiments were carried out in duplicate.

Immunodiffusion tests were carried out at room temperature using the technique of double diffusion in agar plates (Ouchterlony, 1953).

Results

In view of the possible differences in the specificity of antibodies elicited by the same immunogen in different rabbits, the present study was carried out with sera of individual animals. Antibodies directed to the polypeptidyl moieties were measured by using for the precipitation reaction polypeptidyl proteins in which the protein carrier was incapable of reacting with any antibody formed. Thus, *e.g.*, when polypeptidyl-HSA was used for immunization, polypeptidyl conjugates of RSA or RNase were used for detection of the anti-polypeptide antibodies formed.

Sera collected before immunization did not precipitate with any of the immunogens or cross-reacting antigens.

TABLE II: Cross-Reactions of Antisera to Poly-DL-alanyl Proteins with Poly-(DL-, D-, and L-) alanyl Proteins.

Rabbit No.	Immunogen	Precipitant (absorbance/1 ml of serum)[a]		
		Poly-DL	Poly-D	Poly-L
1	p-DL-Ala-RSA (560)	2.10	1.55	0.42
2	p-DL-Ala-RSA (560)	1.16	0.72	0.18
3	p-DL-Ala-RSA (560)	0.97	0.62	0.14
4	p-DL-Ala-RSA (560)	0.50[b]	0.45[b]	0.03[b]
5	p-DL-Ala-BSA (530)	0.78	0.42	0.06
6	p-DL-Ala-BSA (530)	0.51	0.35	0.10
7	p-DL-Ala-BSA (530)	0.32	0.18	0.03
8	p-DL-Ala-HSA (524)	1.75	1.57	0.12
9	p-DL-Ala-HSA (524)	1.02	0.74	0.00
10	p-DL-Ala-HSA (524)	0.82	0.52	0.00
11	p-DL-Ala-HSA (524)	0.36	0.29	0.00
12	p-DL-Ala-HSA (592_s)	1.40	1.18	0.11
13	p-DL-Ala-HSA (592_a)	0.43	0.27	0.00
14	p-DL-Ala-HSA (543)	0.82	0.54	0.00
14	p-DL-Ala-HSA (543)	0.70[b]	0.53[b]	0.00[b]
15	p-DL-Ala-RNase (540)	1.40	1.23	0.00
16	p-DL-Ala-RNase (540)	0.61	0.42	0.06
17	p-DL-Ala-RNase (540)	0.45	0.44	0.00
18	p-DL-Ala-RNase (596)	0.80	0.50	0.03
19	p-DL-Ala-RNase (522)	0.90	0.75	0.05
20	p-DL-Ala-RNase (522)	0.52	0.34	0.00
21	p-DL-Ala-RNase (541)	0.44	0.29	0.03
22	p-DL-Ala-RNase (541)	0.34	0.19	0.06
23	p-DL-Ala-RNase (542)	0.47	0.17	0.04
	Goat			
	p-DL-Ala-RGG (8)	3.75	1.87	0.00

[a] Protein carrier of poly-(DL-, D-, and L-) alanyl conjugates used as precipitants was RSA except for two experiments marked by (b). [b] Protein carrier of the precipitant was RNase.

Peptides used in inhibition studies did not cause any precipitation with IgG preparations isolated either from preimmunization sera or from immune sera.

Specificity of Antibodies Elicited with Poly-DL-alanyl Determinants. Immunization of rabbits with poly-DL-alanyl proteins led to the formation of antibodies directed toward the poly-DL-alanyl determinants, as seen in Table II and Figure 1. These antibodies cross precipitated well with poly-D-alanyl proteins, but gave either no cross-precipitation at all, or only slight cross-precipitation (up to 20%) with poly-L-alanyl proteins. The variation in the specificity of antibody formed by individual rabbits is apparent from the differences in the cross-precipitation with poly-D-alanyl proteins. Thus, *e.g.*, 98% of the anti-poly-DL-alanyl antibodies elicited in rabbit 17 cross-reacted with the poly-D-alanyl determinant, whereas only 69% of the antibodies of rabbit 16 gave such a cross-reaction. When the antiserum of rabbit 14 was treated with polyalanyl conjugates of two different proteins (RSA and RNase), similar amounts of precipitates were obtained in each case. It can also be seen that the extent of cross-precipitation was independent of the protein moiety of the immunogen (see Table II).

The lack of reactivity of anti-poly-DL-alanyl HSA with poly-L-alanyl-RSA is illustrated also in Figure 2A, showing results of an immunodiffusion experiment. In contradistinction, a good precipitin line was obtained upon cross-reaction with poly-D-alanyl-RSA, which showed complete identity with poly-DL-alanyl-RSA.

To elucidate whether the antibodies reacting with either poly-DL-alanyl or poly-D-alanyl proteins are the same, absorption experiments were performed. Anti-poly-DL-alanyl sera, completely absorbed with poly-DL-alanyl proteins, did not further react with poly-D-alanyl (or poly-L-alanyl) proteins. As seen in Table III, when the antisera were completely absorbed with poly-D-alanyl proteins, only limited precipitation occurred upon addition of poly-DL-alanyl proteins, whereas in absorption experiments with poly-L-alanyl proteins, almost all the antibodies were still available for the reaction with poly-DL-alanyl proteins. It can be seen from the data given in Tables II and III that the amount precipitated with the poly-DL-alanyl conjugate after absorption with the poly-D-alanyl conjugate is similar

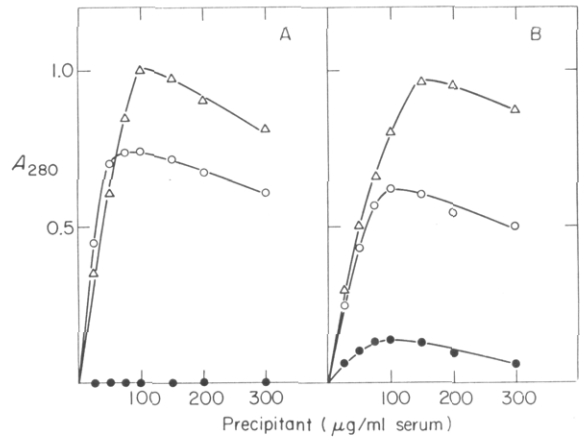

FIGURE 1: Absorbance at 2800 A of solutions in 0·1 N sodium hydroxide (1.1 ml) of precipitates obtained by the addition of poly-DL-alanyl-RSA (560) (△), poly-D-alanyl RSA (551) (○), and poly-L-alanyl-RSA (569) (●) to antisera against: (A) poly-DL-alanyl HSA (524), rabbit 9, and (B) poly-DL-alanyl RSA (560), rabbit 3.

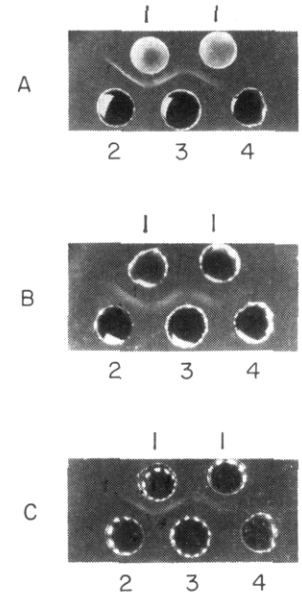

FIGURE 2: Immunodiffusion study of anti-poly-DL-aminoacyl antibodies. (A) 1, anti-poly-DL-alanyl-HSA (524) rabbit 9; 2, poly-D-alanyl-RSA (551); 3, poly-DL-alanyl-RSA (560); 4, poly-L-alanyl-RSA (569). (B) 1, anti-poly-DL-phenylalanyl-HSA (605) rabbit 49; 2, poly-D-phenylalanyl-RNase (592); 3, poly-DL-phenylalanyl-RNase (593); 4, poly-L-phenylalanyl-RNase (591). (C) 1, anti-poly-DL-tyrosyl-RNase (584) rabbit 58; 2, poly-D-tyrosyl-RSA (580); 3, poly-DL-tyrosyl-RSA (581); 4, poly-L-tyrosyl-RSA (545); Concentration of precipitants (in wells 2–4) is 0.1 mg/ml in plate A and 0.05 mg/ml in plates B and C.

to the difference between the amount of antibody precipitated with these two polyalanyl conjugates.

In an earlier study (Schechter *et al.*, 1966) it was shown that poly-DL-alanyl proteins cross precipitate both with anti-poly-L-alanyl and anti-poly-D-alanyl antibodies. These reactions were inhibited by peptides composed exclusively either of L- or of D-alanine. Thus, it was possible to distinguish (by choosing the appropriate inhibitory peptides) between the two antipodal specificities of the antibodies. The cross-precipitations and the inhibition reactions also proved that the poly-DL-alanyl side chains indeed contained sequences of L-alanine residues as well as sequences of D-alanine residues, as predicted from the nature of the polymerization reaction (Katchalski and Sela, 1958). Similar inhibition studies were carried out now with the anti-poly-DL-alanyl system. In order to avoid the degradation of the L peptides by proteolytic enzymes present in the sera, the experiments were carried out with IgG preparations isolated from antisera and devoid of proteolytic activity (Schechter *et al.*, 1966). As apparent from Table IV and Figure 3, peptides of D-alanine were much more efficient inhibitors of the poly-DL-alanyl–anti-poly-DL-alanyl reaction than peptides of L-alanine. L-Alanyl-L-alanine did not inhibit the reaction at all under conditions at which D-alanyl-D-alanine was distinctly inhibitory. In one case, shown in Table IV, total inhibition of the poly-DL-alanyl reaction was obtained with tetra-D-alanine, whereas tetra-L-alanine inhibited only 40% of the reaction. In all the four cases listed in Table IV, tetra-D-alanine (3 μmoles) inhibited

TABLE III: Precipitation of Antipoly-DL-alanyl Antibodies after Absorption[a] with Poly-(D- or L-) alanyl Proteins.

Rabbit No.	Immunogen	Pptn by Poly-DL-alanyl Protein[b] after Absorption with (absorbance/1 ml of serum) Poly-D	Poly-L
3	p-DL-Ala-RSA (560)	0.31	0.78
4	p-DL-Ala-RSA (560)	0	0.42
5	p-DL-Ala-BSA (530)	0.23	0.72
9	p-DL-Ala-HSA (524)	0	1.02
10	p-DL-Ala-HSA (524)	0.04	0.82
13	p-DL-Ala-HSA (592_s)	0.15	0.43
14	p-DL-Ala-HSA (543)	0.12	0.82
18	p-DL-Ala-RNase (596)	0.25	0.80
20	p-DL-Ala-RNase (522)	0.20	0.52
23	p-DL-Ala-RNase (542)	0.28	0.40

[a] Absorption experiments were carried out as described under Materials and Methods. [b] In all experiments RSA was the protein moiety of the precipitant, except for rabbit 4, where RNase was the protein carrier.

TABLE IV: Inhibition of the Precipitin Reactions between Poly-DL-alanyl-RSA and Antibodies to Poly-DL-alanyl Proteins by Tetraalanine Peptides.

Rabbit No.	Immunogen	Inhibition (%) by Tetraalanine: 0.5 μ-mole[a] D_4	0.5 μ-mole[a] L_4	6.0 μ-moles[a] D_4	6.0 μ-moles[a] L_4
3	p-DL-Ala-RSA (560)	61	0	93	32
5	p-DL-Ala-BSA (530)	60	0	90	36
12	p-DL-Ala-HSA (592_s)	72	14	100	40
16	p-DL-Ala-RNase (540)	58	5	86	35

[a] Amount of alanine peptide in the reaction mixture (1.7 ml).

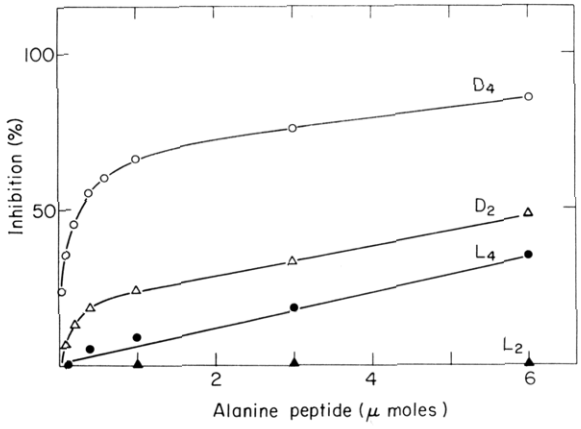

FIGURE 3: Inhibition by alanine peptides of the precipitates obtained by reacting the IgG preparation from antiserum to poly-DL-alanyl-RNase (540) rabbit 16, with poly-DL-alanyl-RSA (560). L_2, D_2, L_4, and D_4 denote di- and tetrapeptides composed either of L- or D-alanine.

completely the poly-D-alanyl–anti-poly-DL-alanyl reaction.

It is known that the region of the antigenic determinant farthest removed from the protein carrier has the major contribution to the antigenic specificity of the antibody formed (Landsteiner, 1945; Karush, 1962; Schechter *et al.*, 1966). As the results reported above show that the antibodies to the poly-DL-alanyl determinant possess mainly anti-poly-D-alanyl specificity, it could be assumed that the poly-DL-alanyl proteins investigated have mainly D-alanine sequences adjacent to the N termini of the polymeric side chains. Even though this seemed improbable in view of the fact that L-alanine sequences as well as D-alanine sequences are similarly distributed in poly-DL-alanine chains, all the polyalanyl proteins listed in Table I were subjected to degradation by leucine aminopeptidase to test this possibility. This enzyme readily attacks bonds between two L-alanine residues, very slowly attacks the L-alanyl-D-alanyl bond, and does not split the peptide bonds between two D-alanine residues (Schechter and Berger, 1966b). A typical chromatographic analysis of digestion mixtures obtained upon treating polyalanyl proteins with LAP is shown in Figure 4 (A–C). Free alanine was found in digests of poly-DL-alanyl and poly-L-alanyl-RSA but not in the digest of poly-D-alanyl-RSA. The amount of free alanine found in digests of poly-DL-alanyl-RSA (560) and poly-DL-alanyl-BSA (530) corresponded to 80% of the amount expected, assuming random copolymerization, and taking in consideration the specificity of the enzyme.

The preponderance of antibodies with poly-D-alanyl specificity in anti-poly-DL-alanyl sera is not limited to rabbits, as apparent from the analysis of a goat antiserum to poly-DL-alanyl-RGG, given in Table II.

Specificity of Antibodies Elicited with Poly-DL-phenylalanyl and Poly-DL-tyrosyl Determinants. In order to find out whether the preferential immune response to D-amino acid sequences upon immunization with antigens possessing poly-DL-aminoacyl determinants is a general phenomenon, we have analyzed two additional systems containing, respectively, peptides of phenylalanine and of tyrosine. In Table V are summarized the specificities of antibodies elicited with poly-L-phenylalanyl and poly-D-phenylalanyl proteins, as well as with poly-L-tyrosyl and poly-D-tyrosyl derivatives. In the polytyrosyl system all rabbits produced stirictly stereospecific antibodies. On the other hand, in the polyphenylalanyl system the immunization of

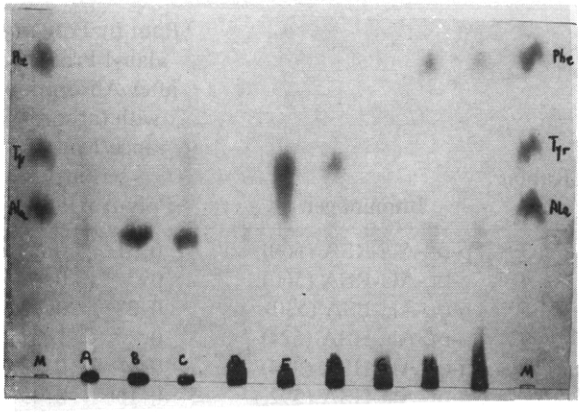

FIGURE 4: Chromatography of reaction mixtures containing LAP (80 μg/ml), 0.05 M sodium-barbital buffer, pH 8.0, 0.005 M $MnSO_4$, and 5% of the following: (A) Poly-D-alanyl-RSA (551); (B) poly-L-alanyl-RSA (569); (C) poly-DL-alanyl-RSA (560); (D) poly-D-tyrosyl-RSA (580); (E) poly-L-tyrosyl-RSA (545); (F) poly-DL-tyrosyl-RSA (581); (G) poly-D-phenylalanyl-RNase (592); (H) poly-L-phenylalanyl-RNase (591); and (I) poly-DL-phenylalanyl-RNase (593). M represents a mixture of alanine, tyrosine, and phenylalanine.

TABLE V: Reaction of Antisera to Poly-L-Phenylalanyl, Poly-D-Phenylalanyl, Poly-L-Tyrosyl, and Poly-D-Tyrosyl Proteins.

Rabbit No.	Immunogen	Precipitant (absorbance/1 ml of serum)[a]		
		Poly-L	Poly-D	Poly-DL
24	p-L-Phe-HSA (588)	0.80	0.10	0.60
25	p-L-Phe-HSA (588)	0.94	0.56	0.90
26	p-D-Phe-HSA (589)	0.30	1.60	1.10
27	p-D-Phe-HSA (589)	0.10	0.75	0.55
28	p-L-Phe-RNase (591)	0.45	0.00	0.30
29	p-L-Phe-RNase (591)	0.33	0.00	0.23
30	p-D-Phe-RNase (592)	0.00	0.52	0.33
31	p-D-Phe-RNase (592)	0.00	0.30	0.20
32	p-L-Tyr-RSA (545)	0.40	0.00	
33	p-L-Tyr-RSA (545)	0.26	0.00	
34	p-D-Tyr-RSA (580)	0.00	0.52	
35	p-D-Tyr-RSA (580)	0.00	0.30	
36	p-L-Tyr-RNase (582)	0.46	0.00	
37	p-L-Tyr-RNase (582)	0.28	0.00	
38	p-D-Tyr-RNase (583)	0.00	0.36	
39	p-D-Tyr-RNase (583)	0.00	0.26	

[a] The precipitants were polyphenylalanyl proteins when the immunogen contained polyphenylalanine, and polytyrosyl proteins when the immunogen contained polytyrosine. The protein moiety of the precipitant was RNase when the immunogen was a modified serum albumin, and RSA when the immunogen was a modified RNase.

TABLE VI: Cross-Reactions of Antisera to Poly-DL-(phenylalanyl or tyrosyl) Proteins with Poly-(DL-, D-, or L-) (phenylalanyl or tyrosyl) Proteins.

Rabbit No.	Immunogen	Precipitant (absorbance/1 ml of serum)[a]		
		Poly-DL	Poly-D	Poly-L
40	p-DL-Phe-RSA (606)	0.64	0.41	0.14
41	p-DL-Phe-RSA (534)	0.52	0.41	0.24
42	p-DL-Phe-BSA (535)	0.73	0.64	0.16
43	p-DL-Phe-BSA (535)	0.62	0.62	0.00
44	p-DL-Phe-BSA (535)	0.64	0.60	0.12
45	p-DL-Phe-HSA (590)	1.42	1.34	0.38
46	p-DL-Phe-HSA (590)	1.30	1.25	0.55
47	p-DL-Phe-HSA (590)	0.75	0.67	0.30
48	p-DL-Phe-HSA (590)	0.68	0.62	0.38
49	p-DL-Phe-HSA (605)	2.50	1.80	0.72
50	p-DL-Phe-HSA (605)	0.97	0.88	0.23
51	p-DL-Phe-HSA (605)	0.42	0.38	0.00
52	p-DL-Phe-RNase (593)	0.64	0.46	0.40
53	p-DL-Phe-RNase (593)	0.53	0.40	0.30
54	p-DL-Phe-RNase (593)	0.39	0.30	0.24
55	p-DL-Tyr-RSA (581)	0.35	0.30	0.07
56	p-DL-Tyr-RSA (581)	0.28	0.26	0.05
57	p-DL-Tyr-RNase (584)	0.36	0.27	0.00
58	p-DL-Tyr-RNase (584)	0.34	0.30	0.00

[a] The precipitants were polyphenylalanyl proteins when the immunogen contained polyphenylalanine, and the polytyrosyl proteins when the immunogen contained polytyrosine. The protein moiety of the precipitant was RNase except for rabbits 52, 53, 54, 57, and 58 where the protein moiety of the precipitant was RSA.

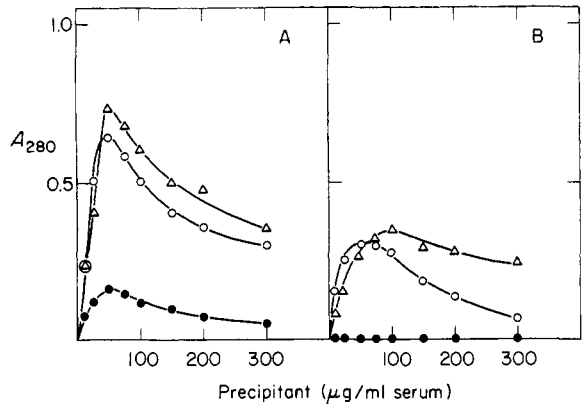

FIGURE 5: Absorbance at 2800 A of solutions in 0.1 N sodium hydroxide (1.1 ml) of precipitates obtained by treating: (A) anti-poly-DL-phenylalanyl-BSA (535), rabbit 42, with poly-DL-phenylalanyl-RNase (593) (△), poly-D-phenylalanyl-RNase (592) (○), and poly-L-phenylalanyl-RNase (591) (●); (B) anti-poly-DL-tyrosyl-RNase (584), rabbit 58, with poly-DL-tyrosyl-RSA (581) (△), poly-D-tyrosyl-RSA (580) (○), and poly-L-tyrosyl-RSA (545) (●).

rabbits with HSA conjugates led to the formation of antibodies which do not seem to be completely stereospecific. This might be due to the fact that the phenylalanine side chains in the immunogen are rather short (see Table I). Thus, the antibodies formed might be directed to the side chain of lysine to which the peptidyl chains are attached, as well.

The immune response toward poly-DL-phenylalanyl and poly-DL-tyrosyl determinants, as well as cross-precipitations with poly-D- and poly-L-aminoacyl conjugates are summarized in Table VI and typical precipitin curves are shown in Figure 5. With both polypeptidyl determinants the antibodies formed reacted better with the appropriate poly-D-amino acid sequences than with the poly-L-amino acid sequences. This finding is corroborated by results of absorption experiments listed in Table VII, as well as by immunodiffusion analyses illustrated in Figure 2B,C.

Similarly to the experiments with polyalanyl proteins, the presence of L-amino acid residues at the N termini of the peptidyl side chains of polyphenylalanyl and polytyrosyl proteins was tested with leucine aminopeptidase. All the polypeptidyl conjugates listed in Table I were checked and typical results are shown in Figure 4D–I. As expected, free phenylalanine was found in digests of poly-DL-phenylalanyl and poly-L-phenylalanyl-RNase but not of poly-D-phenylalanyl-RNase, whereas free tyrosine was found in digests of poly-DL-tyrosyl and poly-L-tyrosyl-RSA but not of poly-D-tyrosyl-RSA.

TABLE VII: Precipitation of Anti-poly-DL-phenylalanyl and Anti-poly-DL-tyrosyl Antibodies after Absorption[a] with Poly-(D- or L-) (phenylalanyl or tyrosyl) Proteins.

Rabbit No.	Immunogen	Precipitation by Poly-DL-(Phe or Tyr) Protein[b] after Absorption with (absorbance/1 ml of serum) Poly-D	Poly-L
40	p-DL-Phe-RSA (606)	0.07	0.32
44	p-DL-Phe-BSA (535)	0	0.48
47	p-DL-Phe-HSA (590)	0.04	0.40
48	p-DL-Phe-HSA (590)	0	0.34
49	p-DL-Phe-HSA (605)	0	1.30
51	p-DL-Phe-HSA (605)	0	0.30
53	p-DL-Phe-RNase (593)	0.05	0.16
54	p-DL-Phe-RNase (593)	0.03	0.14
55	p-DL-Tyr-RSA (581)	0	0.25
56	p-DL-Tyr-RSA (581)	0	0.20
57	p-DL-Tyr-RNase (584)	0	0.29
58	p-DL-Tyr-RNase (584)	0	0.34

[a] Absorption experiments were carried out as described under Materials and Methods. [b] In all experiments RNase was the protein moiety of the precipitant, except for rabbits 53, 54, 57, and 58 where the protein moiety of the precipitant was RSA.

Discussion

The main conclusion to be drawn from the experiments described here is that the specificity of antibodies formed against poly-DL-aminoacyl determinants is predominantly toward sequences of D-amino acid residues. Two earlier observations on the more efficient inhibition of the poly-DL-alanyl immune system with D-alanyl-D-alanine rather than with L-alanyl-L-alanine (Brown *et al.*, 1963), and with a mixture of small peptides of D-alanine but not of L-alanine (Sage *et al.*, 1964), are in agreement with this conclusion.

The preferential response toward D peptides rather than toward L peptides is clearly illustrated by cross-precipitation (Tables II and VI; Figures 1 and 5), absorption (Tables III and VII), immunodiffusion (Figure 2), and inhibition (Table IV and Figure 3) experiments. The preponderance of antibodies with specificity directed toward D-amino acid sequences seems a general phenomenon as it was observed in two animal species (rabbit and goat) and with three different amino acids (Ala, Phe, and Tyr). This type of anti polypeptidyl response is not dependent on the nature of the protein carrier but only on that of the determinant.

Most of the anti-poly-DL-alanyl antibodies had specificity directed against D-alanine sequences. Nevertheless, the existence of antibodies with specificity directed to sequences containing both L- and D-alanine is inferred from the fact that in some sera the sum of

precipitates formed by reacting with poly-D-alanyl and poly-L-alanyl proteins was less than the amount of precipitate formed with poly-DL-alanyl protein (*e.g.*, animals 2, 14, and 23 in Table II). The capacity of anti-poly-DL-alanyl antibodies to interact to some extent with high concentrations of peptides of either D- or L-alanine is apparent from inhibition experiments (Table IV). More cross-reactions with poly-L-aminoacyl determinants were detected in the polyphenylalanyl and the polytyrosyl systems than in the polyalanyl system, possibly owing to the difference in the length of the polypeptide chains attached to the protein. These were much shorter in the conjugates with the aromatic amino acids (Table I) than in those with alanine, and probably involved part of the protein carrier in the specificity determinants.

Polypeptidyl proteins are prepared by the polymerization of *N*-carboxy-α-amino acid anhydrides, using proteins as multifunctional initiators. The ϵ-amino groups of the proteins are mainly responsible for the initiation reaction. The molecular weight distribution of the polypeptidic side chains is relatively sharp (Katchalski *et al.*, 1955). In the polymerization of *N*-carboxy-DL-amino acid anhydrides the rate of formation of LL and of DD bonds is obviously identical, and it may be different from the rate of formation of LD and DL bonds. As the ϵ-amino groups of the protein are not adjacent to asymmetric carbon, there is no reason to assume that there is any steric preference for clustering L- or D-amino acid residues around the initiator. Experimental corroboration of the expectation that poly-DL-alanyl side chains contain sequences both of D- and L-amino acids is found in the cross-precipitation of anti-poly-D-alanyl as well as of anti-poly-L-alanyl antibodies with poly-DL-alanyl proteins (Schechter *et al.*, 1966). A direct proof of the presence of L-amino acids at the N termini of some polymeric side chains was obtained in this study by making use of leucine aminopeptidase (Figure 4).

The question could be asked whether the preferential immune response toward D-amino acid residues is due to the removal of L-amino acid residues enzymatically *in vivo* from the polypeptidyl side chains of the immunogens. Two arguments may be brought againt such a possibility. (a) Poly-L-alanyl side chains in poly-L-alanyl proteins would be expected to be readily digested by enzymes. Nevertheless, poly-L-alanyl proteins are as good immunogens as poly-D-alanyl proteins, leading to the formation of antibodies with specificity directed toward sequences of L-alanine. (b) The action of proteases on poly-DL-amino acyl chains is expected to lead to the formation of polypeptide determinants possessing L-amino acids at the N termini, *i.e.*, in the area of paramount importance for the defining of the specificity of the antibodies formed (Schechter *et al.*, 1966). This is based on the stereochemical specificity of enzymes which can hardly split bonds in which a D-amino acid residue is involved (LD or DL type of bonds) (Dixon and Webb, 1958; Schechter and Berger, 1966b).

The observation that the immunization with poly-DL-aminoacyl proteins results in the formation of antibodies which show mainly anti-D-aminoacyl specificity may be a particular case of the phenomenon of antigenic competition. It seems that determinants composed of L-alanine, D-alanine, or both L- and D-alanine, compete at one of the stages leading to antibody formation, with the D-alanine sequence being the most successful one.

The available information on competition of antigens has been discussed by Adler (1964). The use of well-defined peptide determinants may be of considerable advantage in efforts to understand better this as yet poorly understood immunological response. The study of the antigenic competition making use of polypeptidyl proteins has been recently extended to other model systems (Schechter, 1965).

Acknowledgments

We wish to acknowledge the competent technical assistance of Miss G. Ashkenazi and Miss E. Dascal.

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