ANTIGENICITY OF POLYPEPTIDES (POLY ALPHA AMINO ACIDS)*

XIII. IMMUNOLOGICAL STUDIES WITH SYNTHETIC POLYMERS CONTAINING ONLY D- OR D- AND L-α-AMINO ACIDS

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In continuation of studies to delineate some of the criteria for immunogenicity of synthetic random polymers of α-amino acids, we have investigated the contribution of the optical configuration of the amino acids. It was hoped that by studying the immunogenicity in several species of a variety of random terpolymers containing only D-α-amino acids or mixtures of D- and L-α-amino acids, some definite conclusions could be reached concerning the role of optical configuration in immunogenicity. Previously, we reported that two polymers consisting only of the D-α-amino acids, glutamic acid, alanine, and tyrosine ($G_{60}A_{40}$, $G_{60}A_{30}T_{10}$)[1] were not immunogenic in rabbits or guinea pigs (1), whereas the "isomeric" polymers of L-amino acids were effective immunogens (2, 3). Moreover, the polymer $G_{60}A_{30}T_{10}$, made of D-amino acids, could not act as a carrier in guinea pigs for the haptene specificity of arsanilic acid (4). The polymers employed in this study were optical variations of those previously shown to be immunogenic when made of L-α-amino acids. The findings on immunogenicity will be presented here and the immunochemical relationships of the polymers will be the subject of another publication.

Materials and Methods

Antigens.—Random copolymers were prepared by the polymerization of the appropriate N-carboxy-α-amino acids anhydride of the configuration indicated in Table I, by methods referred to previously (1–5). The average molecular weights were obtained from viscosity measurements as well as from measurements with the analytical ultracentrifuge.[2] Some typical sedimentation patterns of the preparations are shown in Fig. 1.

[1] In this article, G, L, A, and T stand for the amino acids glutamic acid, lysine, alanine, and tyrosine. Subscripts refer to mol per cent amino acid in the polymer. D or L refer to optical configuration of the amino acid.

[2] We acknowledge the assistance of Dr. Ralph Heimer of the Departments of Medicine and Biochemistry with these studies.

TABLE I

Synthetic Polymers of α-Amino Acids Studied for Antigenicity

Polymer	Prepn. No.	Nomenclature employed	Approximate average mol. wt.
L-glu$_{60}$D-ala$_{40}$	M-56	G$_{60}$A$_{40}$ (L, D)	20,000*
D-glu$_{60}$L-ala$_{40}$	M-57	G$_{60}$A$_{40}$(D, L)	25,000*
L-glu$_{42}$D-lys$_{28}$L-ala$_{30}$	M-53	G$_{42}$L$_{28}$A$_{30}$ (L, D, L)	22,000‡
L-glu$_{42}$L-lys$_{28}$D-ala$_{30}$	M-52	G$_{42}$L$_{28}$A$_{30}$ (L, L, D)	30,000‡
D-glu$_{42}$L-lys$_{28}$L-ala$_{30}$	M-50	G$_{42}$L$_{28}$A$_{30}$ (D, L, L)	21,000‡
D-glu$_{42}$D-lys$_{28}$D-ala$_{30}$	M-54	G$_{42}$L$_{28}$A$_{30}$ (D, D, D)	70,000‡
D-glu$_{56}$D-lys$_{38}$D-tyr$_6$	M-36B	G$_{56}$L$_{38}$T$_6$ (D, D, D)	21,000*
D-glu$_{36}$D-lys$_{24}$D-ala$_{35}$D-tyr$_5$	M-58	G$_{36}$L$_{24}$A$_{35}$T$_5$ (D, D, D, D)	15,000*

* Values obtained from viscosimetric analyses.
‡ Values obtained from ultracentrifuge measurements.

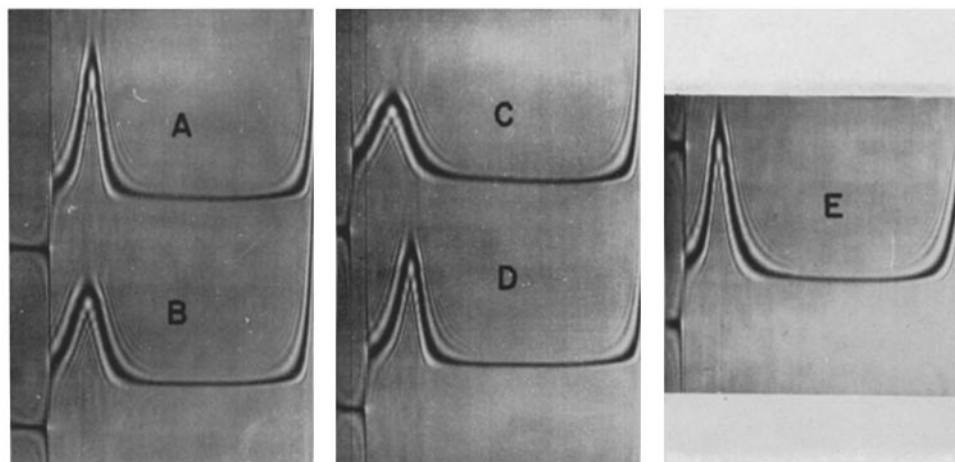Fig. 1. Ultracentrifuge patterns of 1 per cent solution (pH 7.5 0.1 M phosphate buffer) of G$_{42}$L$_{28}$A$_{30}$ polymers. Analyses performed in Spinco model E ultracentrifuge at 56,100 RPM, phase plate angle 50°.

Pattern A, L, D, L polymer (160 minutes); pattern B, L, L, L polymer (160 minutes); pattern C, D, L, L polymer (80 minutes); pattern D, D, D, D polymer (80 minutes); pattern E, L, L, D polymer (80 minutes).

Immunization and Bleeding.—

Rabbits: New Zealand white rabbits weighing between 2.5 and 3 kg were immunized with the polymers in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit) for 3 courses as described previously (2). Each rabbit received a total of 60 mg of polymer (50 mg adjuvant, 10 mg solution). Bleedings were taken weekly for 3 weeks after each immunization.

Guinea pigs: Male and female Hartley Strain guinea pigs weighing 300 to 450 gm were used. Antigen solutions were emulsified with an equal volume of complete adjuvant so that

the immunizing dose of 100 μg was present in 0.5 ml. This was injected into the four foot-pads on day 0 and again 3 and 6 weeks after the initial injection. On days 7 and 14, after each immunization, the animals were bled from the retroorbital sinus and then skin-tested with 100 and 10 μg of the copolymer in 0.1 ml. Reactions were read at 4 to 6 and 24 hours. The early (immediate) reactions were graded according to the severity of the edema and hemorrhage. The diameters of the induration of erythema of the late (delayed) reactions were recorded.

Mice: Swiss-Webster mice were injected intradermally with 1 mg of polymer ($G_{36}L_{24}A_{35}T_{5}$) in complete adjuvant in 0.2 ml. 2 weeks later, this was repeated. Bleeding from the retroorbital plexus was performed 9 days after this latter injection.[3]

Humans: The method of immunization employed was similar to that used in a previous report (6). After withdrawal of 50 ml of blood, the volunteers were skin-tested with 0.1 ml of polymer solution (10 mg/ml). Reactions were read after 15 to 20 minutes and after 24 hours. The individuals (12 per polymer) were injected intramuscularly on 2 subsequent days with the appropriate polymer solution. A total of 10 mg was injected. The bleedings and skin testings were repeated 3 weeks after the last injection. All sera were preserved by the addition of 1:10,000 merthiolate and kept frozen until studied. The human sera contained, in addition as preservative, 0.25 per cent phenol.

Testing of Sera.—

Passive cutaneous anaphylaxis (PCA): The PCA test was performed on all rabbit and guinea pig sera (0.1 ml undiluted serum or dilutions thereof) as described by Ovary (7), using a challenging injection of 250 μg of polymer. Blueing reactions were recorded 20 minutes later.

Agar diffusion: The Preer double diffusion in agar technique was employed in screening all guinea pig and rabbit sera that were positive by PCA (8). Undiluted sera were tested with polymers containing 2.5, 5, 10, 20, and 40 μg N per ml. Tubes were placed in a cold room and observed for several weeks. Reactions were graded 0 to 4+ based on the intensity of the band or bands as in a previous report (9).

Quantitative precipitin studies: The microprecipitin technique for analysis of human sera as developed by Heidelberger and MacPherson was used (10). 3.0 ml of aliquots of serum and 2 to 3 concentrations of antigen (1.5, 4.0, 15 μg polymer N) were set up at 0°C and observed for 7 to 10 days. The tubes were mixed daily. At the end of this period, the tubes were centrifuged and any precipitates that formed were analyzed for antibody by the Folin-Ciocalteu reagent.

Passive hemagglutination: The Takatsy micromodification for passive hemagglutination (HA) of tannic acid–treated and antigen-coated sheep red blood cells was employed (11, 12). All sera, *i.e.* rabbit, guinea pig, mouse and human, with the exception of those obtained from animals injected with polymers of $G_{60}A_{40}$, were tested as previously described (2). Polymers that did not contain lysine have been shown not to adsorb directly to tanned sheep red blood cells. Hemagglutination noted in dilutions of serum greater than 1:4 were considered significant.

Fluorescence microscopy: Hyperimmune rabbit antisera against the polymers $G_{55}L_{43}T_{6}$, $G_{42}L_{28}A_{30}$, $G_{60}A_{40}$, and $G_{36}L_{24}A_{35}T_{5}$ and the sera from the exsanguination bleedings of rabbits immunized with the D forms of the polymers were fractionated with 50 per cent ammonium sulfate (5°C for 2 hours). The globulin precipitate was dialyzed free of sulfate and conjugated with fluorescein isothiocyanate according to the procedure of Marshall *et al.* (13). Unbound flour was removed by dialysis and absorption with rabbit liver powder. Brush imprints on microscopic slides were made of spleens. These were stained with the appropriate fluorescent conjugate according to the sandwich technique; *i.e.*, overlaying first with polymer, removing the unbound polymer, and staining with the homologous or heterologous conjugated antiserum

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[3] These experiments were conducted by Dr. Paul Pinchuck.

TABLE II

Summary of Immune Responses in Several Species against Synthetic Polymers

Preparation	Species injected	No. of reactors
$G_{60}A_{40}$ (L, D)	Rabbit	0/10*
	Guinea pig	0/10‡
$G_{60}A_{40}$ (D, L)	Rabbit	0/10*
	Guinea pig	0/10‡
$G_{42}L_{28}A_{30}$ (L, D, L)	Rabbit	5/10*, §
	Guinea pig	7/12‡, §
$G_{42}L_{28}A_{30}$ (L, L, D)	Rabbit	2/6*, §
	Guinea pig	5/10‡, §
$G_{42}L_{28}A_{30}$ (D, L, L)	Rabbit	2/6*, §
	Guinea pig	2/17‡, §
$G_{42}L_{28}A_{30}$ (D, D, D)	Rabbit	0/6*, §
	Guinea pig	0/12‡, §
$G_{56}L_{38}T_{6}$ (D, D, D)	Rabbit	0/6*, §
	Guinea pig	0/10‡, §
	Man	0/12‖, §
$G_{36}L_{24}A_{35}T_{5}$ (D, D, D, D)	Rabbit	0/10*, §
	Guinea pig	0/10‡, §
	Man	0/12‖, §
	Mouse	0/10§

* Values are based on the passive cutaneous anaphylaxis reactions in guinea pigs.
‡ Values are based on PCA and skin testing.
§ Values are based on passive hemagglutination testing.
‖ Values are based on quantitative precipitin reactions and skin testing.

(14). Slides were examined with a Leitz ortholux microscope using as light source an Osram HBO 200 mercury vapor lamp.[4]

RESULTS

Table II summarizes the results obtained with the polymers. It is evident that the polymers containing only D-α-amino acids were not immunogenic in any of the species studied. Similarly, the $G_{60}A_{40}$ polymers (L, D and D, L) produced no detectable responses in rabbits or guinea pigs. The only polymers that did produce an immune response in rabbits and guinea pigs were those of the $G_{42}L_{28}A_{30}$ series containing at least 2 L-α-amino acids. The summary of the

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[4] These studies were carried out by Mr. Robert Orlando.

nature of the responses is given in Table III. All sera that were positive by PCA were positive by passive hemagglutination, and sera negative by PCA did not agglutinate antigen-coated cells.[5] The strongest sera in any group as measured by precipitin and PCA reactions also gave the highest HA titers.

$G_{42}L_{28}A_{30}$ (L,D,L)—The best immunogen in this group for both rabbits and guinea pigs was the L,D,L terpolymer. 5/10 rabbits reacted with good responses. The sera from these rabbits gave 4+ reactions in agar diffusion, had

TABLE III

Immune Reactions against Polymers of $G_{42}L_{28}A_{30}$ Series Containing D and L-α-Amino Acids

Configuration of amino acids in $G_{42}L_{28}A_{30}$	Rabbits					Guinea pigs									
	PCA*,‡ response		Agar* diffusion		HA§ titer	Cutaneous reactions				PCA ‖ response		Agar* diffusion		HA§ titer
						Immediate		Delayed						
	Course		Course		Course III	Course		Course		Course		Course		Course
	II	III	II	III		I	II	I	II	I	II	I	II	II
L, D, L	2000 (4) 100¶	2500 (5) 125¶	3+ (4)	3+ (5)	16–32	6	9	6	4	6	6	2+ (5)	2+ (6)	16–32
L, L, D	250 (2) 12.5¶	200 (2) 10¶	1+ (2)	1+ (2)	4–8	5	5	5	1	5	5	1+ (2)	1+ (2)	16–32
D, L, L	50 (2) 2.5¶	50 (2) 2.5¶	1+ (1)	1+ (1)	2–4	2	2	5	5	1	1	0	0	8

* Values in parenthesis refer to numbers included in average.
‡ Values refer to dilution of serum giving unquestionable PCA reaction.
§ Reciprocal of serum dilution giving definite hemagglutination pattern with positive sera.
‖ Sera not titrated. Values refer to number of animals reacting.
¶ Average antibody values (μg AbN/ml serum) based on PCA sensitivity of 0.05 μg AbN/ml serum.

HA titers of 8 to 32, and elicited average PCA titers of 2000. 7/12 guinea pigs reacted well against this polymer as evidenced by positive PCA reactions and also positive reactions of their sera in agar diffusion.

$G_{42}L_{28}A_{30}$ (L,L,D and D,L,L).—The order of decreasing immunogenicity with these polymers as shown in both the rabbit and guinea pig data was L, L, D; D, L, L. With the former polymer, although the sera of 2/6 rabbits reacted by PCA, they gave very weak precipitin reactions. Similarly, of the 5 guinea pig sera positive by PCA, only 2 gave positive reactions in agar. The D, L, L polymer produced very poor responses in both species studied. Of the 2/6 rabbits re-

acting, only 1 serum reacted by agar diffusion, whereas none of the guinea pig sera gave a positive reaction in agar diffusion.

The cross–PCA reactions among the $G_{42}L_{28}A_{30}$ polymers and the respective antisera are presented in Table IV.

Fluorescence Microscopy.—Brush smears of spleens from rabbits immunized with the L form of the polymers stained strongly with the homologous conjugated antiserum and indicated the presence of large numbers of antibody-producing cells. This reaction could be blocked by first treating with unconjugated antiserum. However, there was no indication of specific antibody-forming cells in the rabbits injected with the isomeric D form of the polymer. The same

TABLE IV

Extent of Cross PCA Reactions Among $G_{42}L_{28}A_{30}$ Polymers and Respective Rabbit Antisera*, ‡

Configuration of $G_{42}L_{28}A_{30}$ polymer used in preparing antiserum	Configuration of $G_{42}L_{28}A_{30}$ polymer injected				
	L, L, L,	L, D, L	L, L, D	D, L, L	D, D, D
L, L, L	4+§	+	+	−	−
L, D, L	+	4+	+	±	−
L, L, D	±	−	3+	−	−
D, L, L	−	−	−	2+	−

* Reactions based on use of undiluted serum and challenging injection of $250\mu g$ of polymer.

‡ Values based on average diameter and intensity of blueing reaction.

§ Reaction of similar intensity could be obtained using 1:100 dilution of homologous serum and challenging injection of $50\mu g$ homologous polymer.

negative findings were obtained when staining was performed with the conjugates of the anti-L form of polymer as well as with serum from animals immunized with the all D form of the polymer.

DISCUSSION

The results reported here confirm our previous observations that random copolymers consisting only of D-α-amino acids are not immunogenic in any of the species tested (1). These findings are based not only on the analysis of antibody in sera, but also from the fluorescent microscopy studies indicating that even at the cellular level there is no indication of formation of specific antibody.

The same polymers, when made of L-amino acids ($G_{42}L_{28}A_{30}$, $G_{36}L_{24}A_{35}T_5$, G_{56} $L_{38}T_6$), are effective antigens in rabbits, guinea pigs, mice, and humans and can carry haptene specificity for guinea pigs. Others have also shown that polymers of D-amino acids are non-immunogenic (15–17). These findings are compatible with our previous discussion (4, 5), and the concept of others that the metabolism of the antigen (18, 19), previous to the formation of the immunogenic fragment carrying the antigenic determinant, is a necessary but not sufficient cri-

terion for immunogenicity. It is believed that this cleavage may be performed by proteolytic enzymes present in macrophages (20) and may be blocked by D-amino acid polymers. The following observations noted in our laboratory may have significance in this regard. Groups of rabbits that did not respond to the polymers of D-amino acids ($G_{60}A_{40}$, $G_{60}A_{30}T_{10}$, $G_{42}L_{28}A_{30}$, $G_{36}L_{24}A_{35}T_5$) or to the $G_{60}A_{40}$ (L, D) and (D, L) had reduced responses against subsequent immunizations with the isomeric polymer made of the L-amino acids. Also an unpublished finding related to our studies on immunological tolerance indicates that injections of D-glu$_{60}$ D-ala$_{40}$ into newborn rabbits causes a significant depression of the immune response in the adults against the L isomer, $G_{60}A_{40}$. Whether any specific inhibition of the enzymic (proteolytic) systems of the animal's macrophages has occurred is being studied now. In addition to the requirement for antigenicity of the degradation by tissue enzymes, it appears that the degradation product must undergo other metabolic steps to induce an immune reaction (21). Possibly the presence of D-amino acids (not of the haptene kind) may inhibit these necessary steps.

The recent report of Gill *et al.*, that a polymer of $G_{55}L_{39}T_6$ (D, D, D) is immunogenic in rabbits (22), is in sharp contrast to the findings of this publication. Gill *et al.* have stated that the immunogenicity was about 1/4 to 1/3 that of the L-amino acid polymer. What is puzzling about this report is the almost consistent finding that there was little indication of any rise in antibody levels after booster injections of antigen. In fact, there was an almost consistent decrease in antibody levels in 7/7 animals having detectable antibody.

It is also surprising to note that, although these authors indicate that the incorporation of D-tyrosine into the non-immunogenic $G_{60}L_{40}$ (D, D) polymer leads to the formation of an immunogenic material, the antibody is inhibited significantly by D-lysine. In fact, the inhibition by D-lysine was said to be as effective in the above system as L-lysine for the antibody against $G_{55}L_{38}T_6$ (L, L, L). In view of the conflicting findings of Gill *et al.* and the possible odd interactions of synthetic polymers with serum proteins (23), it would have been of great interest to learn what type of immunoglobulin was involved in the interaction. Also, it is of importance to know whether any biological activity could be ascribed to the antibody in addition to its precipitation by polymer. In our studies (2, 24), and in those of Ben Efraim *et al.* (25), PCA was an excellent indication of the immune response in rabbits. If, indeed, antibody against the $G_{55}L_{39}T_6$ (D, D, D) were produced in the amounts of 20 to 126 μg antibody N per ml of serum, unquestionable PCA and passive systemic anaphylaxis reactions could have been produced in guinea pigs. In this same publication, as well as in a more recent report, Gill and coworkers agree that the metabolizability of the polymer is important for immunogenicity and that their metabolic studies indicate the unique presence of D-amino acid proteases and peptidases (26). However, the metabolic studies with I^{131}-labeled polymers do not sub-

stantiate their conclusions. The serum elimination patterns of the D and L popypeptides were exactly the same. Moreover, the I^{131} found in the urine was all dialyzable and no information was presented as to whether this iodide was associated with tyrosine or peptide-like material. The implication that the up-take of polymer by macrophages (15 to 25 per cent uptake of both L and D forms of $G_{58}L_{36}T_6$) is indicative that the polymer will be metabolized is incorrect, as it has been shown that haptene conjugates of poly-D-lysine are taken up by macrophates (21) as well as carbon particles and other inert materials without being degraded. It is also known that iodinated proteins and peptides can be deiodinated leading to the presence of iodide in the urine. Our own *in vitro* stud-ies dealing with the degradation of synthetic polymers by rabbit spleen extracts and proteolytic enzymes, such as pepsin, trypsin, and pronase, indicate that the polymers consisting solely of D-amino acids are not degraded at all (27). The findings of Levine and Benacerraf that only conjugates of poly-L-lysine, but not those of poly-D-lysine, are degraded *in vitro* (by spleen extracts) and *in vivo* are in agreement with all similar studies so far save those of Gill *et al.*

The $G_{42}L_{28}A_{30}$ and $G_{60}A_{40}$ polymers, containing D- and L-amino acids, were prepared to learn the effect of the introduction of a D-amino acid into polymers which, in the L form, were good immunogens. Although some physical data to be reported separately indicated that the various optical isomers of $G_{42}L_{28}A_{30}$ and $G_{60}A_{40}$ were indeed different, it was important to establish their immuno-chemical uniqueness and exclude the possibility of impurities of $G_{42}L_{28}A_{30}$ (L, L, L). Although weak cross-reactions were noted among the various antisera (PCA reactions) and isomeric $G_{42}L_{28}A_{30}$ preparations (Table IV), none of the samples, save the homologous L, L, L preparation precipitated with high titered rabbit and guinea pig anti-L-glu$_{42}$ L-lys$_{28}$ L-ala$_{30}$ sera, indicating the absence of L, L, L polymer. The most immunogenic polymer in the series of $G_{42}L_{28}A_{30}$ and $G_{60}A_{40}$ was the $G_{42}L_{28}A_{30}$ (L, D, L). The polymeric sequences of L-glutamic acid and L-alanine similar to those present in the good immunogen $G_{60}A_{40}$ may have contributed to the effective immunogenicity of the L, D, L polymer. That such may be the case is indicated by the observed cross-reaction of $G_{60}A_{40}$ with anti-$G_{42}L_{28}A_{30}$ sera of L, D, L, configuration. Although the latter polymer was a fair antigen, the introduction of D-lysine resulted in changes in its specificity. When a D-amino acid was present in the other poor immunogens (L, L, D and D, L, L polymers) immunogenicity was not enhanced. With these polymers, it appears that the immunogenicity is related to the number and type of sequences contributed by the L-amino acids, although some specificity can be attributed to the D-amino acid in the same way as a haptene contributes to specificity. The observation of Sela *et al.* that $G_{50}A_{40}T_{10}$ (L, L, D) was a good immunogen in rab-bits with specificity directed towards D-tyrosine (16) is analogous to our findings with the $G_{42}L_{28}A_{30}$ (L, D, L) polymer. The poor immunogens (L, L, D and D, L, L polymers) have random sequences contributed by D-glu and L-ala or L-glu and

D-ala. These polymers alone were shown above not to be immunogenic. The reason for this reduced immunogenicity is not known. However, it is believed that with these polymers the presence of a number of repeating glutamyl residues of L configuration is important for immunogenicity, and the introduction of the D-amino acid reduced the digestibility of the polymer and possibly the formation of the proper sized antigenic determinant. Our most recent observation that polyglutamic itself can be an effective immunogen in rabbits (28) when the technique of Plescia *et al.* (29) is employed is consistent with this idea.[6] Although we have previously stated that polyglutamic acid is not immunogenic in the several species studied (30–32), it appears that changing the physical state of the polyglutamic acid by reaction with methylated bovine serum albumin altered sufficiently the handling of the polymer by the host's cells to allow formation of the requisite size of glutamyl groupings to act as determinants for antibody formation. The introduction of alanine into polyglutamic acid to form $G_{60}A_{40}$, a good immunogen which causes formation of antibody having a large cross-reaction with polyglutamic acid (31), may have altered the handling of the polymer in an analogous manner. This finding of the immunogenicity in rabbits of polyglutamic acid also helps explain our reported observation that injections of polyglutamic acid into neonatal rabbits produced tolerance against subsequent immunization with $G_{60}A_{40}$ (5).

SUMMARY

It has been demonstrated that polymers consisting solely of D-α-amino acids are not immunogenic in rabbits, guinea pigs, man, and mouse, whereas the same polymers of L-α-amino acids are very effective antigens. This has been attributed to the importance of metabolizability of a polymer in contributing to its immunogenicity. In the glu-lys-ala series of polymers, the immunogenicity of a polymer of 2 L-amino acids and a D-amino acid appears to be governed by the immunogenicity of the 2 L-amino acids. However, some of the specificity may be directed towards configurations containing the D-amino acid. It has been noted that injections of rabbits with polymers of D-amino acids has resulted in a reduced response against the isomeric L polymer.

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