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Antigenicity of Polypeptides (Poly Alpha Amino Acids). X. Studies with Polymers of D Amino Acids.* (28423)

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Several groups have studied the antigenicity polymers of L-α-amino acids. The polymers have been either of the "straight chain" linear random copolymer(1,2) or the multichain type(3,4). Investigations have indicated that the *copolymers* of L-α-amino acids in contrast to the homopolymers of a single amino acid(5) may be antigenic in rabbits, (5-9), guinea pigs(10,11) and man(12). The use of synthetic polymers also afforded insight into some of the structural factors controlling immunogenicity of proteins. To learn more about these parameters, the antigenicity of copolymers consisting of 2 and 3 D amino acids has been studied in both rabbits and guinea pigs. This report presents data which indicate that polymers made solely of D amino acids have little or no immunogenicity.

Materials and methods. The copolymers studied for antigenicity are listed in Table I. They were prepared by the random polymerization of mixtures of the N-carboxy anhydrides of the amino acids which were present in the mol percent as indicated in the final product. Before use, the solution of the polymer was dialized to remove any low molecular weight materials. Polymerization was allowed to go to completion. The weight average molecular weight was estimated from viscosity measurements(13). Nomenclature for the polymers is as follows: G, A and T refer to glutamic acid, alanine and tyrosine respectively and subscripts refer to the mol percent composition of the amino acid in the random copolymers. The optical configuration of the polymers is indicated by (D) or (L) preceding the polymer. The arsanilic acid derivative of D(GAT), (As(D)(GAT)), was prepared by Dr. B. Benacerraf. Five mg of the polymer were treated with an excess of diazotized arsanilic acid. The mixture was stirred for 1 hour, then dialyzed for several days to remove unreacted arsanilic acid. Arsanyl groups were determined spectrophotometrically at 333 mμ using the mono arsanyl derivative

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TABLE I. D Amino Acid Polymers Studied for Immunogenicity.

Copolymer	Ratio amino acids	Nomenclature	Wt avg molecular wt
glu ala	60:40	(D) $G_{60}A_{40}$	47,000
glu ala tyr	60:30:10	(D) $G_{60}A_{30}T_{10}$	33,000

of chloroacetyl tryosine as a standard. The molar extinctive coefficient was 15,400. Analyses indicated the presence of 16.2 arsanyl groups per molecule.

Rabbits. Twelve New Zealand white rabbits weighing between 2.5 and 3 kg were immunized with either the (D)$G_{60}A_{40}$ or (D) $G_{60}A_{30}T_{10}$ polymer in a complete Freund's adjuvant. The solutions of polymers at a concentration of 80 mg per ml were emulsified with an equal volume of Arlacel C + Bayol F present in a ratio of 9:1(14,15). Heat killed *M. butyricum* were added to a final concentration of 1 mg/ml. Rabbits were bled before immunization, then injected in the toe pads with the adjuvant mixture containing 20 mg polymer. They were bled 2 and 3 weeks after the first injection (Course I). They then received another adjuvant injection of 20 mg of polymer and were bled on the 2nd, 3rd and 4th weeks after the injection (Course II). After a rest of about a month, the rabbits were given an intradermal injection of the adjuvant mixture containing 10 mg of polymer and an intravenous injection of a solution of the polymer (1 ml of 10 mg/ml). One week later, they were bled again (Course III). Immunization and bleeding as described above with the D polymers were repeated a week later in the same rabbits with polymers of similar composition, but consisting of L amino acids.

Guinea pigs. Eight to 10 Hartley strain albino guinea pigs weighing 300-350 g were injected with each polymer or derivative. Immunization was performed with the polymers in complete adjuvants. Guinea pigs were injected in the footpads with the adjuvant mixture containing 200 μg of polymer or 100 μg of the arsanilic acid derivative of (D)$G_{60}A_{30}T_{10}$. They were skin tested on days 7, 14 and 21. Those injected with (D)$G_{60}A_{40}$ and (D)$G_{60}A_{30}T_{10}$ were tested with 50 μg of homologous polymer, and those receiving As(D)$G_{60}A_{30}T_{10}$ were tested with 10 μg derivative. Skin reactions were read after 4, 6 and 24 hours. Immunization and skin testing were then repeated. Six weeks after first injection, the guinea pigs were bled.

All sera were preserved by addition of 1:10,000 'Merthiolate' and kept frozen until studied.

Passive cutaneous anaphylaxis. The PCA test was performed on all sera as described by Ovary(16). One-tenth ml of undiluted serum or appropriate dilutions thereof were injected intradermally into guinea pigs weighing 200 to 250 g. Three hours later, 1 ml of a solution containing 250 μg of the copolymer or appropriate homopolymer and 0.5% Evans blue dye solution was injected intravenously. The bluing reactions were observed 20 minutes later. When questionable reactions occurred, the test was repeated by first injecting dye alone.

Micro precipitin test. To 1 ml of the sera, 1-2 μg N of the suitable polymer was added. The tubes were mixed, incubated at 37°C for ½ hour and placed in the cold. They were observed daily for precipitation for 4-5 days.

Results. The copolymers of D amino acids failed to cause an immune response detectable in any of the rabbits or guinea pigs by means of PCA or qualitative micro precipitin tests. While none of the rabbits responded to the D amino acid polymers, 6 and 8 rabbits responded to reimmunization with (L)$G_{60}A_{40}$ and (L)GAT respectively as measured by PCA. Guinea pigs immunized with As(D) $G_{60}A_{30}T_{10}$ did not react either to the carrier polymer or hapten group.

Although the D polymers did not precipitate with antibody produced against the L polymers, and did not inhibit the homologous precipitin reaction(17), they did react with respective antisera in the PCA reaction (Table II). The L α and D α forms of polyglutamic acid (G_{100}) also reacted by PCA reaction.†

Discussion. The data presented here with polymers of D amino acids indicate the importance of metabolizability of a high mole-

† The author wishes to thank Dr. J. Kovacs for supplying poly-D-glutamic acid.

TABLE II. Passive Cutaneous Anaphylaxis Reactions of Polymers with Rabbit Antisera.*

	(L) $G_{60}A_{40}$ antiserum #3† Polymer tested			(L) $G_{60}A_{30}T_{10}$ antiserum #3† Polymer tested		
	(D) $G_{60}A_{40}$	(L) G_{100}	(D) G_{100}	(D) $G_{60}A_{30}T_{10}$	(L) G_{100}	(D) G_{100}
Undil	28	28	24	28	31	19
1-10	23	24	17	20	21	4
1-100	19	20	15	5	18	0
1-200	15	15	5	0	11	0

* Values refer to average diameter of bluing reaction after injection of 250 μg polymer.
† Sera reacted to 1-1000 dilution with homologous antigens.

cular weight polymer towards its immunogenicity. The data of Gill *et al.*(18), on the non-antigenicity in rabbits of a copolymer of D glutamic acid and D lysine (D)$G_{60}L_{40}$ also support this idea. This relationship between immunogenicity of an antigen and its ability to be degraded was first expressed by Wells(19). Later information has indicated that the ability of a highly polymerized material to be degraded is a *necessary*, but *not sufficient* criterion for immunogenicity. Also, there is the distinction between ability of a polymer to be antigenic *per se* and its ability to act as a hapten when coupled to either an antigenic or non-antigenic, but metabolizable, backbone structure. The data supporting these ideas are as follows: The L homopolymers poly glu(20) and poly lys(21) and copolymers of L amino acids (α linkage)(22) are susceptible to enzymes such as pepsin, trypsin, papain, pronase, etc. Peptides of D amino acids are not degraded by the usual proteolytic enzymes(23). As pointed out by Campbell, non-digestible artificial polymers such as polyvinyl pyrrolidone (PVP) and native non-digestible materials such as gum acacia and colloidal dyes are not antigenic (24). Actually a very high molecular weight preparation of PVP exhibited limited antigenicity in humans(25) but similar PVP preparations were not antigenic in rabbits or guinea pigs(26). Also, a copolymer of N vinyl pyrrolidone and vinyl acetate was not antigenic in rabbits or guinea pigs(26). The possible importance of fragmentation or digestion of antigen in antibody formation has been discussed by Campbell and Garvey(27) and Rittenberg and Nelson(28).

The other example of the antigenicity in rabbits of an unnatural polymer is that of the D γ glutamyl polypeptide of *B. mesentericus*, *B. anthracis* and *B. subtillis*(29). The purified polymer itself was not antigenic and it was necessary to inject whole bacteria to evoke antibodies to the D-γ-glutamyl polypeptide. When the D-γ-glutamyl polypeptide was injected into humans, only 1/24 produced a significant amount of antibody (5.6 μg AbN/ml serum)(30). When a hapten is associated with a D amino acid polymer, (D) $G_{60}A_{30}T_{10}$, no antibody was produced, in contrast to the results with haptens coupled to digestible antigenic or non-antigenic backbone(31,32). Examples are the immunogenicity in guinea pigs of the conjugate of poly L lysine and dinitrobenzene(33) and of azo phenyl arsonate when coupled to polytyrosyl gelatins(34), (L)$G_{60}A_{30}T_{10}$(32) or poly L-tyrosine(35). When haptenic groups such as azo benzene arsonate or dinitrobenzene are coupled to non-digestible polymers such as polystyrene, or a copolymer of styrene and p-acetoxystyrene(36), no antibody is formed.

Here, too, there may be an exception in the observations of Micheel and Petersen on the immune response in rabbits (detected by anaphylactic shock) against synthetic antigens of polyvinyl amine onto which blocks of tyrosine and glucosamine (alone or together) have been polymerized(37). It was not possible to test the serum for antibody because the polymer caused non-specific precipitation of serum proteins. These results are of uncertain significance since the polymer has an exceedingly high cationic charge and is toxic. The charge effect which caused non-specific precipitation of serum proteins *in vitro*, may have produced the same precipitation *in vivo* which led to the interpretation of anaphylactic shock.

The PCA reaction of (D)$G_{60}A_{40}$ and (D) $G_{60}A_{30}T_{10}$ with antisera against the respec-

tive L amino acid polymer is of interest. The reaction was not detectable by *in vitro* precipitation, inhibition of precipitation or by quantitative complement fixation techniques (17). Similar negative results have been reported by Gill *et al.*, with (D) glu lys polymers (precipitation and inhibition)(18) and by Sage *et al.* with polyalanine, (D, DL and L) linked to bovine serum albumin(38). These latter workers used the quantitative complement fixation reaction and noted the unique serological specificity of the isomeric forms of the polyalanyl peptides. In the results reported here with the D polymers, reactions were noted only by PCA. It is also significant that the polyglutamic acid compounds (D and L) reacted with both antisera by the PCA reaction.

Ovary has shown that an antigen or hapten must be at least bivalent to provoke the PCA reaction(39). This indicates that although the D polymers have a helical sense opposite from the L polymers, the free carboxyl groups (which are involved to a considerable degree in the antigenic specificity of the polymers) are so oriented they can approach to some degree the appropriate antibody site, interact and mediate the reactions leading to extravasation of the Evans blue dye. However, the forces of interaction must be weak as the PCA reaction is the only evidence of this fleeting interaction.

Summary. It has been shown that random copolymers of D amino acids (gluala, glu ala tyr) are not antigenic in rabbits or guinea pigs. A hapten coupled to a D amino acid polymer was not immunogenic. This lack of immunogenicity has been ascribed to the inability of unnatural synthetic polymers to be degraded by known enzymes. The interaction of the polymers of D amino acids with antisera against the L forms of the same polymer was detectable only by the PCA reaction. Reasons for the non-immunogenicity of the D polymers and their interactions with antibody have been discussed.

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Electrocardiographic Changes Preceding Catecholamine-Induced Arrhythmias.* (28424)

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A lengthening of the QT interval of the electrocardiogram preceding onset of ventricular fibrillation was reported by Chenoweth during a study of epinephrine-hydrocarbon syncope(1). The increase in the QT interval was observed in tracings in which there was a regular unifocal beat before the development of fibrillation. Since epinephrine by itself may increase the QT interval(2), the present study was undertaken to determine the significance of this electrocardiographic change prior to onset of experimental ventricular arrhythmias. In animals treated with hydrocarbons relatively small doses of epinephrine or norepinephrine may precipitate ventricular fibrillation while isoproterenol causes only cardioacceleration(3). The changes in the QT interval produced by these catecholamines were therefore compared in animals predisposed to arrhythmias by administration of petroleum ether.

Methods. Lead II of the electrocardiogram was recorded in 20 cats anesthetized with sodium pentobarbital (30 mg/kg). Control responses to intravenous injection of 5-10 μg/kg epinephrine, norepinephrine or isoproterenol were first observed. Ventricular arrhythmias were induced 15 minutes later using the method of Garb and Chenoweth(3) in which 0.1 ml/kg petroleum ether (B.P. 30-60°C) was injected through an endotracheal tube and the same dose of catecholamine was repeated 30 seconds later. Episodes of epinephrine-induced ventricular arrhythmias are usually transient in cats under pentobarbital anesthesia(4). It was therefore possible to repeat the injections of petroleum ether and catecholamines in most animals.

The PR, QRS, and QT intervals were measured during the various phases of the experiments. Because of the inverse relationship between the duration of the QT interval and the heart rate in various metabolic states (5), the ratios of these intervals to the corresponding cardiac cycle lengths were calculated.

Results. Epinephrine caused a progressive lengthening of the QT interval after injection of petroleum ether and prior to the onset of ventricular tachycardia and fibrillation (Fig. 1). This prolongation of the QT interval was not observed after epinephrine during the initial control period.

Norepinephrine and epinephrine produced similar changes in the electrocardiogram after petroleum ether. In the tracings showing multiple focal ventricular tachycardia or fibrillation after petroleum ether and epinephrine, there was a 28% increase in the QT interval compared to that produced by norepinephrine in the animals in which a regular sinus rhythm was maintained. However, the same dose of isoproterenol injected 30 seconds after petroleum ether did not produce any

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