Immunochemical Studies on the Poly-γ-D-glutamyl Capsule of *Bacillus anthracis*. I. Characterization of the Polypeptide and of the Specificity of Its Reaction with Rabbit Antisera*

Joel W. Goodman and Danute E. Nitecki

ABSTRACT: Capsular polypeptide from a strain of *Bacillus anthracis* was isolated and found to consist almost exclusively of D-glutamic acid with possible traces of aspartic acid; hexose content, as determined by reaction with orcinol, was negligible. The molecular weight of the polypeptide, as estimated by terminal amino end group analysis using trinitrobenzenesulfonic acid, was 33,500 ± 3800. Antisera which precipitated with the purified polypeptide were elicited in rabbits by repeated injections of suspensions of encapsulated organisms.

The precipitating element in these sera was found to be in the γ-globulin fraction. A specificity for residues of D-glutamic acid was demonstrated by the inability of a sample of poly-α-L-glutamic acid to either precipitate with the antisera or to appreciably inhibit precipitation of the antisera with the homologous anthrax polypeptide. Of a number of synthetic peptides and polypeptides, those which contained residues of D-glutamic acid were much more effective inhibitors of the homologous precipitin reaction than those which contained L- but no D-glutamic acid. The eight possible dipeptides and four branched tripeptides of glutamic acid were tested for their capacity to inhibit the homologous precipitin reaction. The best inhibitor of these proved to be a branched tripeptide consisting of a residue of L-glutamic acid substituted at both carboxyl groups with residues of D-glutamic acid. This tripeptide was markedly superior to a branched tripeptide consisting only of D-glutamic acid.

The capsular polypeptides of organisms of the genus *Bacillus* are apparently composed exclusively of residues of glutamic acid (Hanby and Rydon, 1946; Pongor, 1950) linked by γ-peptide bonds (Bruckner and Kovács, 1957). While the polypeptide from *Bacillus anthracis* seems to consist only of D-glutamic acid, preparations of capsular material from *Bacillus subtilis* have been reported to contain both D and L isomers of the amino acid (Bovarnick, 1942; Thorne *et al.*, 1954).

It was realized as early as 1937 that these polymers of glutamic acid are involved in the immune specificity of the bacteria (Ivanovics and Bruckner, 1937). The precipitin reactions of horse and rabbit antisera, prepared by immunization with suspensions of killed organisms, with the homologous polypeptides (Ivanovics, 1940) and with a series of synthetic glutamyl polypeptides (Bruckner *et al.*, 1958; Hanby *et al.*, 1950; Ivanovics, 1958; Utsumi *et al.*, 1959), have been studied and the findings disclosed a specificity for residues of D-glutamic acid linked by γ-peptide bonds in the anthrax antisera.

The technique of hapten inhibition of the precipitin reaction has been used to establish the extents of the combining regions of dextrans involved in immunochemical reactions, using oligosaccharides of glucose as inhibitors (Kabat, 1958). Dextrans are eminently suitable for studies of this nature because of their relative structural simplicity, and in the same sense the polyglutamic acid immune system is ideal for investigating the extent of the combining region on a protein antigen.

In the present communication, data concerning the properties of a capsular polypeptide isolated from a strain of *Bacillus anthracis*, the serologic specificity of its reaction with rabbit antisera, and hapten inhibition of the homologous immune system using amino acids and synthetic di- and tripeptides of glutamic acid will be presented.

Materials and Methods

Bacillus anthracis Polypeptide. A virulent strain of *B. anthracis*, designated M-36, was obtained from Dr. Curtis B. Thorne. When cultured on a solid medium in an atmosphere of 30 parts of CO_2 to 70 parts of air (Thorne and Leonard, 1958) for 48 hr, a thick mucoid growth was obtained. Upon microscopic examination the bacilli showed extensive encapsulation. The organisms were harvested and the capsular polypeptide was isolated according to the procedures described by Hanby and Rydon (1946).

Antisera. Rabbits were given an injection of an anthrax spore vaccine (American Cyanamid Co.) 1–2 weeks prior to injection of encapsulated organisms. A thick suspension of anthrax strain M-36 was heated

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in a boiling-water bath for 1 hr. This treatment destroyed the vegetative cells but some spores survived as was evidenced by growth upon prolonged incubation in liquid medium. The complete destruction of spores required autoclaving, a procedure which sharply diminished the immune response of rabbits to the polypeptide. Animals were given three intravenous injections per week of a suspension of M-36 containing about 20–25 mg dry wt of bacteria. After a series of 12 injections, the animals were rested for 3–4 weeks and the course was repeated. Of 48 rabbits immunized in this fashion, 16 responded with levels of antibody to the glutamyl capsular polypeptide which were detectable by precipitin analysis. These animals were given additional courses of injections and were bled by cardiac puncture five days after the last injection of each course. Additional bleedings were taken twice weekly until the quantity of precipitating antipolypeptide antibody dropped below useful levels. Sera from the rabbits were pooled.

Amino Acids and Peptides. A complete series of chromatographically pure D- and L-amino acids was obtained from Mann Chemical Co. The following peptides were synthesized and graciously provided by Dr. Joseph Kovács and his colleagues: γ-D-Glu-Gly.[1] γ-L-Glu-Gly, α-*t*-butyl-γ-D-Glu-Gly, α-*t*-butyl-γ-L-Glu-Gly, poly-γ-D-Glu-Gly, poly-γ-L-Glu-Gly, poly-β-D-Asp, poly-β-L-Asp, poly-γ-D-Glu-β-Ala, poly-γ-L-Glu-β-Ala. The synthesis and properties of the poly-γ-D-Glu-Gly and poly-γ-L-Glu-Gly have been described (Kovács *et al.*, 1964); their molecular weights, determined by terminal amino end group analysis using fluorodinitrobenzene, were between 1000 and 2000. The molecular weight of the poly-γ-D- and -L-glutamyl-β-alanines was approximately 10,000 per polymer, determined by sedimentation velocity. The molecular weights of the polyaspartic acids are uncertain. Dinitrophenyl end group and Van Slyke nitrogen analyses indicated values of 7000–10,000 but sedimentation velocity experiments suggested weights of 1300–2500. Further evaluation is in progress. These data were provided by Dr. Kovács. A sample of synthetic poly-α-L-glutamic acid with an average molecular weight of 66,000 was kindly donated by Dr. Paul H. Maurer.

The complete series of eight possible dipeptides and four branched tripeptides (in which one amino acid residue is substituted at both carboxyl groups) of glutamic acid were synthesized in this laboratory and obtained in very homogeneous form as shown by elementary, electrophoretic, chromatographic, and amino end group analyses. Their synthesis, purification, and characterization are described elsewhere (Nitecki and Goodman, 1966).

γ-Globulin. An aliquot of the pool of rabbit antisera was used for the preparation of γ-globulin by precipitation with sodium sulfate (Kekwick, 1940). The product obtained from three successive precipitations was then chromatographed on carboxymethylcellulose (Carl Schleicher and Schull Co.). Following removal of other serum proteins from the column with 0.01 M phosphate buffer, pH 6.0, γ-globulin (γG)[2,3] was eluted with 0.01 M phosphate buffer plus 0.3 M sodium chloride, pH 8.0. This product proved to be pure γG when tested by immunoelectrophoresis (Scheidegger, 1955) with goat antiserum against rabbit serum proteins (Hyland Laboratories).

Immunodiffusion and Immunoelectrophoresis. The methods of Ouchterlony (1953) and Scheidegger (1955) were employed for immunodiffusion and immunoelectrophoretic analyses, respectively.

Hexose. Reaction with orcinol was used for determination of hexose in samples of purified M-36 polyglutamic acid, as described by Winzler (1955). Glucose was used as standard.

Amino Acid Analysis. Samples of purified M-36 polyglutamic acid were hydrolyzed in 6 N HCl for 6 hr at 110° in sealed tubes. Amino acid analysis was performed using a Beckman Model 120B analyzer as described by Spackman *et al.* (1958).

Molecular Weight Estimates. The molecular weight of M-36 polyglutamic acid was determined by amino-terminal end group labeling using 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) (Okuyama and Satake, 1960). Aqueous solutions of glutamic acid in concentrations ranging from 0.015 to 0.50 μmole/ml were prepared. One milliliter of these solutions was mixed with 1.0 ml of 4% sodium bicarbonate solution and 1.0 ml of a 0.1% solution of TNBS. The reaction mixtures were kept in the dark at 37° for 2 hr, at which point 1.0 ml of 1 N HCl was added and the optical densities at 340 mμ immediately were measured. The concentration range was rather limited due to high blank values (OD = 0.4–0.6), presumably attributable to picric acid formation, but a reasonably good Beer–Lambert relationship was obtained. Determinations of the molecular weight of the glutamyl polypeptide were performed using solutions of polypeptide ranging from 0.125 to 10 mg/ml. After correction for the blank value, the concentration of trinitrophenylamino group was read directly from the standard plot for glutamic acid, assuming identical extinction coefficients for derivatives of glutamic acid and the polypeptide. This assumption appears warranted since the extinction coefficients of di- and tripeptides of glutamic acid seem to be identical (Nitecki and Goodman, 1966). The calculation of molecular weight is based on one primary amino group per molecule.

Optical Rotation. The optical rotations of amino acids and hydrolyzed peptides and polypeptides were measured in an O. C. Rudolph and Sons Model 80 polarimeter equipped with an oscillating polarizer and a photoelectric attachment with a voltage-stabilizer (Raytheon). The light source was a Rudolph Model 620

[1] The peptide nomenclature is that described by Young (1962).

[2] The nomenclature used is that recommended by the Conference on Immunoglobulin Nomenclature, *Bull. World Health Organ. 30*, 447 (1964).

[3] Abbreviations used: γG, γ-globulin; TNBS, 2,4,6-trinitrobenzene-1-sulfonic acid.

sodium light. Solutions of amino acids in 6 N HCl were measured in a 2-dm tube with a 3-mm bore. M-36 polyglutamic acid as well as controls of L-glutamic acid were dissolved in 5 ml of 6 N HCl, placed in sealed tubes, and hydrolyzed for 5 hr in an autoclave at 120°. Each sample was then filtered through a pad of 50-mg of acid-washed Norit, freshly prepared for each sample.

Paper Chromatography. Acid hydrolysates of peptides were chromatographed on Whatman No. 1 filter paper using the following solvent systems: pyridine–water–acetic acid (50:15:30); 1-butanol–acetic acid–water (120:50:30); methyl ethyl ketone–acetic acid–water (70:30:25). Spots were developed with ninhydrin.

Electrophoresis. For qualitative analysis of peptides, samples were applied to Whatman No. 1 filter paper and electrophoresed at 35 v/cm in each of two solvents (Katz *et al.*, 1959): (1) 10 ml of acetic acid and 1 ml of pyridine diluted to 200 ml with water, pH 3.5–3.7; (2) 10 ml of pyridine and 0.4 ml of acetic acid diluted to 200 ml with water, pH 6.4–6.5. In some instances, preparative electrophoresis was performed using Whatman No. 3 paper under the same conditions. Strips containing the desired material were cut out and eluted with water and the eluates were lyophilized.

Quantitative Precipitin Tests. The pool of rabbit antisera was assayed for its ability to precipitate with the homologous purified poly-γ-D-glutamyl capsular peptide and with the synthetic polypeptides. The pH of all peptide solutions was adjusted to 7.3–7.7 prior to use in precipitin tests. The antiserum (0.2 ml) was used and total volumes of all quantitative precipitin determinations were adjusted to 1.0 ml with saline. After 6–7 days in the refrigerator, the washed precipitates were analyzed for protein colorimetrically by the Folin-Ciocalteau tyrosine method (Heidelberger and MacPherson, 1943). Standards of rabbit γ-globulin were analyzed simultaneously to provide specific optical density values. The purified M-36 capsular polypeptide did not react with the Folin-Ciocalteau reagent.

Quantitative Assays of Inhibition by Peptides. The ability of various amino acids and peptides to inhibit precipitation of antiserum by the homologous polypeptide was determined. Solutions of the peptides were adjusted to pH 7.3–7.7 prior to use. Known amounts of peptides were added to 0.2 ml of serum containing about 90 μg (0.64×10^{-3} μmole, assuming a molecular weight of 140,000) of precipitable antipolypeptide antibody. After incubation at room temperature for 30–60 min, a suitable quantity of antigen was added to each tube and to control tubes which contained antiserum but no inhibitor. Final volumes were 1.1 ml. The tubes were refrigerated for 6–7 days and the washed precipitates were analyzed for protein colorimetrically by the Folin-Ciocalteau tyrosine method (Heidelberger and MacPherson, 1943) in a total volume of 5.0 ml. Percentage inhibition was calculated from the difference in protein precipitated in the presence and in the absence of added hapten.

Passive Cutaneous Anaphylaxis. The capacity of purified M-36 polypeptide to elicit typical passive cutaneous anaphylaxis reactions (Ovary, 1958) was assayed in guinea pigs. Volumes (0.1 ml) of rabbit antiserum to *B. anthracis* M-36, diluted to contain about 5 μg of antibody protein/ml, were injected intradermally into the shaved abdomens of guinea pigs weighing about 350 g. A total of four injection sites were used on each animal. Control sites in each animal received injections of saline or serum from rabbits prior to immunization with anthrax. Eighteen hours later the animals received an intravenous injection of 0.1 mg of M-36 polypeptide in an aqueous solution of 1% Evans blue dye. After another period of 30 min the skins were retracted and the presence or absence of dye in the injected areas was noted. The control areas showed uniformly negative reactions.

Results

The polypeptide isolated from strain M-36 of *Bacillus anthracis* was characterized for purity and several physicochemical properties and the data are summarized in Table I. Acid hydrolysates of the polypeptide yielded only glutamic acid, as shown by high-voltage electrophoresis and paper chromatography. Amino acid analysis of the polypeptide using the Beckman automatic amino acid analyzer also showed only glutamic acid. Four analyses were run and a trace of aspartic acid appeared in one of the chromatograms. It amounted to less than 0.5% glutamic acid and, at that level, was of questionable significance. The other three chromatograms gave negative results for all amino acids other than glutamic acid.

TABLE I: Properties of Capsular Polypeptide Isolated from *B. anthracis* Strain M-36.

Constituents (%)	
Glutamic acid	$\geqq 99.0$
Other amino acids	<0.5
Hexose	<0.5
Specific optical rotation of hydrolysate (deg)	-29.8
Molecular weight	$33,500 \pm 3800$

The hexose content of the polypeptide was also negligible. No hexose was detected using the orcinol method and the sensitivity of the method precluded the presence of more than 0.5% hexose in the polypeptide.

The specific optical rotation of the hydrolyzed polypeptide was −29.8°, in reasonable agreement with the accepted value of −31.5° for D-glutamic acid. The specific optical rotation values for several concentrations of L-glutamic acid controls identically treated varied from + 29.0° to +31.0°. The concentration of glutamic acid in the samples of hydrolyzed polypeptide was determined by a quantitative ninhydrin reaction.

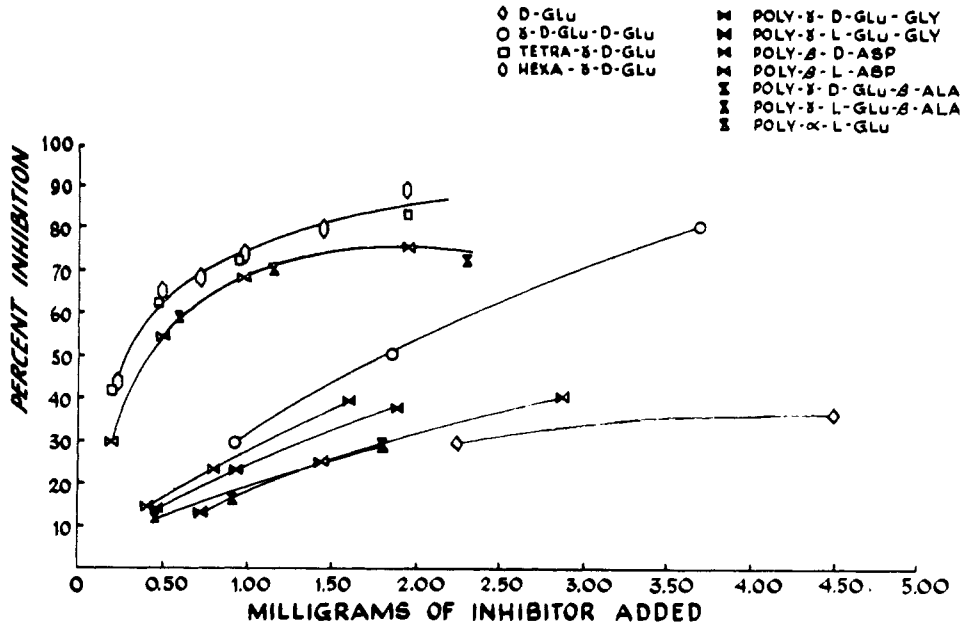

FIGURE 1: Inhibition of the precipitin reaction between 5 μg of M-36 glutamyl polypeptide and 90 μg of antipolypeptide antibody by various peptides and polypeptides.

A value of 33,500 ± 3800 for the molecular weight of the polypeptide was derived by reaction with TNBS. Six separate determinations were carried out and the standard deviation reflects the variance of the values which were secured.

By quantitative precipitin analysis, the pool of rabbit antisera was found to contain about 450 μg of antibody protein/ml of serum when tested with purified polypeptide. This system gave a typical precipitin curve which exhibited zones of antibody excess, equivalence, and antigen excess in which the amount of precipitate steadily declined as the quantity of antigen rose. When the γ-globulin from a sample of the serum pool was separated from the other serum proteins, the precipitating factor was almost entirely contained in the γ-globulin fraction, which was shown to be pure γG by immunoelectrophoresis. The polypeptide, however, did not precipitate with either unfractionated antiserum or purified γG when tested in semisolid medium (agar), using the Ouchterlony double-diffusion and immunoelectrophoretic techniques. The reason for this inability to precipitate in agar is unknown but may be due to an interaction between the acidic polypeptide and a constituent of the medium.

In passive cutaneous anaphylaxis tests, well-defined reactions were obtained in guinea pigs when 0.5 μg of antipolypeptide antibody was injected intradermally and 0.1 mg of polypeptide was introduced intravenously. While the assay is extremely sensitive, the absence of carbohydrate and amino acids other than glutamic acid by the analytical methods employed, which would have detected as little as 1% of such constituents, makes it extremely probable that the skin reactions were caused by the polypeptide immune system.

No precipitation was observed when the antiserum was tested with poly-α-L-glutamic acid (mol wt 66,000), poly-γ-D-Glu-Gly, poly-γ-L-Glu-Gly, poly-γ-D-Glu-β-Ala, poly-γ-L-Glu-β-Ala, poly-β-L-Asp or poly-β-D-Asp in amounts ranging from 10 μg to 10 mg. A volume of serum was used which gave a precipitate consisting of 90 μg of antibody protein precipitable by 5 μg of M-36 polypeptide.

Assays of the various polypeptides for activity in inhibiting the precipitin reaction between rabbit antiserum and homologous M-36 polypeptide are shown in Figure 1. The quantity of inhibitor is calculated on a weight basis rather than on a molar basis, as molecular weights were not known with precision and because of the probable multivalency of the polypeptides. It is felt that the absence of precipitability is due to weakness of reactivity rather than univalency of these materials. Of the polypeptides tested, poly-γ-D-Glu-Gly and poly-γ-D-Glu-β-Ala were easily the most effective, each giving 50% inhibition with less than 0.5 mg, about 100 times the quantity of M-36 polypeptide used. The polypeptides which lacked residues of D-glutamic acid were much poorer, none giving 50% inhibition with the maximum amounts tested. Poly-α-L-Glu is not shown in the figure, but with quantities of 2.25, 4.50, and 9.04 mg the corresponding inhibitions produced were 29, 26, and 48%. For comparison, curves for D-Glu, γ-D-Glu-D-Glu, and commercially obtained preparations of tetra- and hexapeptides (Cyclo Chemical Co.) of γ-D-Glu are shown. The commercial peptides were found to be heterogeneous mixtures when analyzed by electrophoresis and chromatography, containing glutamic acid and intermediates of the synthetic process.

They are shown merely to indicate that peptides of higher molecular weight than the dipeptide gave increased inhibition. The dipeptide, on the other hand, was a better inhibitor on a weight basis than any of the polymers lacking D-glutamic acid. Poly-β-D-Asp was significantly superior to poly-β-L-Asp but much poorer than the polymers containing D-glutamic acid.

The results of inhibition assays with amino acids and smaller synthetic peptides are graphically summarized in Figure 2. Of the amino acids shown, D- and L-glutamic acids were the best inhibitors, about 25 μmoles of each giving 50% inhibition. Surprisingly, the two optical isomers were identical in inhibitory capacity, unlike the polymers containing either of the two isomers; L-glutamine was poorer than L- or D-glutamic acid on a molar basis as 20 μmoles of the amide gave only 29% inhibition while the same quantity of the amino acids gave about 40% inhibition. The aspartic acids gave relatively weak inhibition; 30 μmoles of the D isomer produced 19% inhibition while the same quantity of the L isomer gave 15% inhibition. A number of other D- and L-amino acids were tested, but only D-lysine is shown in Figure 2 in order not to clutter this illustration unnecessarily. All the other amino acids tested gave results similar to those obtained with aspartic acid and lysine.

The dipeptides γ-D-Glu-Gly and γ-L-Glu-Gly were both considerably poorer inhibitors than any of the glutamyl dipeptides. γ-D-Glu-Gly was more effective than γ-L-Glu-Gly but not significantly different from the blocked derivative α-*t*-butyl-γ-D-Glu-Gly. On the other hand, α-*t*-butyl-γ-L-Glu-Gly was a markedly weaker inhibitor than γ-L-Glu-Gly.

Of the glutamyl dipeptides, those with D-glutamic acid at the free amino end were stronger inhibitors than those with L-glutamic acid at that position, with the exception of α-D-Glu-L-Glu, which together with α-L-Glu-L-Glu were the poorest inhibitors of this group. The quantities of inhibitor required for 50% inhibition are given in Table II. Thus, γ-D-Glu-D-Glu, α-D-Glu-D-Glu, and γ-D-Glu-L-Glu all required less than 7 μmoles to cause 50% inhibition of the precipitin reaction. α-D-Glu-L-Glu, which is the only one of the four dipeptides with D-glutamic acid at the amino-terminal position to differ from the homologous dipeptide in two ways (α-peptide bond and L-Glu at the carboxyl-terminal position), needed 11.5 μmoles for the 50% inhibition level. α-D-Glu-D-Glu was somewhat superior to γ-D-Glu-D-Glu at lower inhibition levels, but this situation was reversed above about 70% inhibition. As can be seen from Figure 2 and Table II, most of the dipeptides were closely bunched in inhibitory capacity.

The four dipeptides with L-glutamic acid at the amino-terminal position all required more than 7 μmoles to give 50% inhibition of the precipitin reaction (Figure 2, Table II). Those with a residue of D-glutamic acid were better than the two containing only the L isomer and α-L-Glu-L-Glu, which bears the least resemblance to γ-D-Glu-D-Glu of the four in terms of structure and configuration, was the weakest inhibitor.

Of the four branched tripeptides which were synthesized and assayed for inhibitory capacity, α,γ-L-Glu-(D-Glu)$_2$ was by far the most effective and markedly better than α,γ-D-Glu-(D-Glu)$_2$ (Figure 2, Table II). Only 2.9 μmoles of α,γ-L-Glu-(D-Glu)$_2$ was needed for 50% inhibition while 5.0 μmoles of α,γ-D-Glu-(D-Glu)$_2$ was required for the same degree of inhibition. The other two tripeptides were about as effective as the best dipeptides on a molar basis. None of the peptides produced significant inhibition of an unrelated immune system, rabbit antibody to bovine serum albumin and the homologous antigen, when tested at the same levels as in the polyglutamyl system, demonstrating that activity in the latter system was specific.

TABLE II: Quantities of Peptide Required for 50% Inhibition of the Homologous γ-D-Glutamyl Polypeptide–Antipolypeptide System

Inhibitor	Quantity for 50% Inhibition (μmoles)
γ-D-Glu-D-Glu	6.7
α-D-Glu-D-Glu	5.8
γ-D-Glu-L-Glu	6.7
α-D-Glu-L-Glu	11.5
γ-L-Glu-D-Glu	7.7
α-L-Glu-D-Glu	8.7
γ-L-Glu-L-Glu	9.8
α-L-Glu-L-Glu	11.5
α,γ-D-Glu-(D-Glu)$_2$	5.0
α,γ-L-Glu-(D-Glu)$_2$	2.9
α,γ-D-Glu-(L-Glu)$_2$	6.0
α,γ-L-Glu-(L-Glu)$_2$	6.7

Molecular models of α,γ-D-Glu-(D-Glu)$_2$, α,γ-L-Glu-(D-Glu)$_2$, and γ-D-Glu-γ-D-Glu-D-Glu were constructed to determine if any relationships might be apparent between the latter two which could account for the activity of α,γ-L-Glu-(D-Glu)$_2$. The models were oriented such that all the amide bonds were *trans* and the carboxyl groups were as far apart as possible, since at a pH of 7.5 they should be extensively ionized. No obvious similarities between the linear tripeptide and α,γ-L-Glu-(D-Glu)$_2$ or concomitant differences between it and α,γ-D-Glu-(D-Glu)$_2$ which could explain the differences in inhibitory activity between the two branched tripeptides were discernible.

Although, as stated previously, the commercial preparations of tetra- and hexa-γ-D-glutamic acid were highly impure, results obtained with them in inhibition tests are shown in Figure 2 for purposes of comparison. Whether plotted on a weight basis, as in Figure 1, or on a molar basis, as in Figure 2, they were more active than any of the other peptides tested. Of course, calculation of molar quantities involves the assumption of a single molecular species, which in this instance is not the case. However, since these preparations were also the most active on a weight basis, the results suggest that larger peptides of glutamic acid than those tested in this study

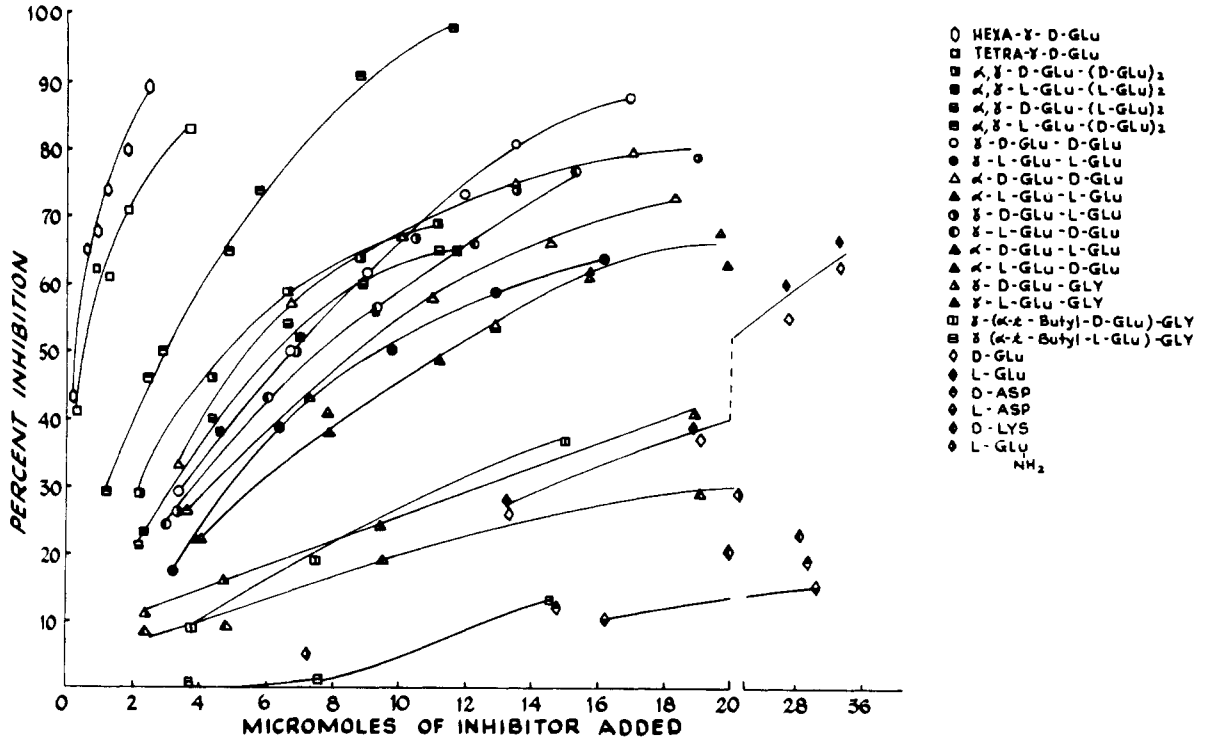

FIGURE 2: Inhibition of the precipitin reaction between 5 μg of M-36 glutamyl polypetpide and 90 μg of antipolypeptide antibody by various amino acids and peptides. Please note the following corrections in nomenclature: □□ = α-*t*-butyl γ-D-Glu-Gly; ⊟ = α-*t*-butyl-γ-L-Glu-Gly.

will produce increased inhibitory activity. In fact, since the impurities in the commercial preparations probably represent intermediates in the synthetic process, the activities shown are probably lower than would be given by pure tetra- and hexapeptides. The preparation of a series of glutamyl peptides of increasing molecular weight is presently in progress.

Discussion[4]

An extensive series of investigations covering the period 1937–1959 established the existence of antibodies in rabbits and horses which were specific for the glutamyl polypeptide capsules of organisms of the genus *Bacillus* (Ivanovics and Bruckner, 1937; Ivanovics, 1940; Bruckner *et al.*, 1958; Hanby *et al.*, 1950; Ivanovics, 1958; Utsumi *et al.*, 1959). Specificity appeared to be established by studies of the cross-reactivity of the antisera with synthetic polypeptides of defined composition and structure (Bruckner *et al.*, 1958; Hanby *et al.*, 1950; Ivanovics, 1958). Thus, of synthetic poly-γ-D-, poly-γ-L-, poly-α-L-, and poly-α-D-glutamic acids, only poly-γ-D-glutamic acid could precipitate with antiserum against *Bacillus anthracis*. A more recent report has demonstrated that under the proper conditions nonspecific precipitation between basic serum proteins such as lysozyme and acidic substances such as glutamyl polypeptide or nucleic acids may occur (Leonard and Thorne, 1961). Leonard and Thorne were unable to demonstrate antibody specific for glutamyl polypeptide in the sera of rabbits which had been immunized with encapsulated *Bacillus anthracis* although the sera would precipitate with polypeptide; the precipitating factor could be removed by other acidic polymers such as deoxyribonucleic acid (DNA). Indeed, Sela and Steiner (1963) have shown that the enzymatic activity of lysozyme could be inhibited by acidic polypeptides which formed small aggregates with the enzyme. The inhibition was more effective at pH values below 6 than at higher values.

Several findings presented in this investigation support the antigen–antibody nature of the precipitating system involving glutamyl polypeptide and rabbit antiserum. (1) The quantitative precipitin curve of the reaction between rabbit antiserum and purified polypeptide showed characteristic regions of antibody excess, equivalence, and antigen excess. (2) γ-Globulin from immune serum was isolated and shown to be pure γG by immunoelectrophoresis. This fraction contained almost all the precipitating activity with purified polypeptide. (3) The antisera were unable to precipitate with a synthetic poly-α-L-glutamic acid which had a molecular weight of approximately 66,000, about twice that of the homologous polypeptide. The α-L polymer was also an extremely poor inhibitor of the precipitin reac-

[4] In conversation with Dr. Michael Sela, we have become aware of the possibility that proteolytic enzymes in serum may influence the results of quantitative inhibition assays of synthetic peptides. The effects of rabbit serum and specific proteolytic enzymes on the peptides used in this investigation are currently being studied.

tion between antiserum and the γ-D polymer; 9.04 mg of poly-α-L produced only 48% inhibition of the reaction given by 5 μg of poly-γ-D. (4) In inhibition tests with synthetic polymers containing D- or L-glutamic acid, the polymers with the D isomer were much more effective than polymers similar in all respects save the substitution of L-glutamic acid for D-glutamic acid. (5) Purified poly-γ-D-glutamic acid was capable of eliciting passive cutaneous anaphylaxis reactions in guinea pigs which had been sensitized with immune serum. As far as is known, the capacity to sensitize tissue in this manner is peculiar to γ-globulin.

The antigenicity of polypeptides has also been the subject of much recent investigation. A variety of copolymers of L-amino acids have been found to be immunogenic in several species (Maurer, 1962; Gill and Doty, 1962; Sela, 1962). However, polymers composed of a single amino acid have in most instances been shown to be incapable of stimulating antibody formation (Maurer, 1957; Buchanan-Davidson *et al.*, 1959). In one study in which a high molecular weight polymer of glutamic acid was injected into humans, one of 24 subjects responded with a level of antibody which could be detected by serologic means (Ostroff *et al.*, 1958). Homopolymers, on the other hand, if coupled to protein carriers, can elicit antibodies which have specificity directed against the polyamino acid portion of the complex (Sage *et al.*, 1964; Arnon *et al.*, 1965a). In earlier studies of the anthrax polypeptide–antipolypeptide system, attempts to induce antibody formation using purified polypeptide were unsuccessful (Ivanovics, 1940). It therefore seems likely that the immunogenicity of the polypeptide is dependent upon the presence of other, more complex substances and it may thus behave much like a hapten. Its immunogenicity is very weak, as evidenced by the need for long courses of injections of large quantities of antigen in order to obtain useful levels of antibody and the fact that only about 25% of the animals responded with such levels.

Marked differences in immunogenicity have been noted between polyamino acids and polypeptides of different optical configuration. Maurer (1965) found that polymers consisting solely of D-α-amino acids were not immunogenic in rabbits, guinea pigs, humans, or mice, whereas the same polymers of L-α-amino acids were very good antigens. Similarly, Parker *et al.* (1965), studying the immunogenicity in guinea pigs of L- and D-polylysines substituted with penicilloyl, dinitrophenyl, and tosyl groups, found that the L-polylysine derivatives produced an antibody response in about 20% of the animals tested, while the D-polylysine conjugates gave uniformly negative results. Similar results were obtained by Levine (1964). In contrast to these findings, Gill and his co-workers (1964) reported that a high molecular weight copolymer of three D-amino acids was antigenic in rabbits. In another study using L- and D-polyalanines coupled to bovine serum albumin as antigens, antisera were produced against the D-polyalanine conjugates and the antibodies exhibited very pronounced stereospecificity (Sage *et al.*, 1964). The findings presented here support the idea that antibodies specific for determinants comprised solely of D-amino acids can be elicited, but they shed no light on the antigenicity of such polypeptides when used in pure form, as reported by Gill and his collaborators. Our results differ from those of Sage *et al.* in that their antisera to D- and L-polyalanines displayed a very rigid stereospecificity (Sage *et al.*, 1964) while the antisera to poly-γ-D-glutamic acid did react to varying degrees with peptides and polymers which contained L- but no D-glutamic acid (Figures 1 and 2). The possibility of racemization during synthesis of the peptides is not supported by analytical data (Nitecki and Goodman, 1966).

In a study involving peptide inhibition of the precipitin reactions between copolymers and their homologous antisera, Gill *et al.* (1963) concluded that ionic interactions were of prime importance in the specificity of acid or basic polypeptides. In the anthrax polypeptide immune system, several pieces of evidence appear to minimize the role of carboxyl groups in the specificity of the polypeptide. Thus, α-*t*-butyl-γ-D-Glu-Gly, which has the α-carboxyl group substituted and, therefore, the glutamyl component has no free carboxyl groups, was about as effective an inhibitor as γ-D-Glu-Gly (Figure 2). Furthermore, aspartic acid was much poorer than glutamic acid and no better than lysine, which bears an opposite charge. It would appear from these data that the carboxyl group is not a critical factor in the specificity of this polypeptide.

The inability of the rabbit antianthrax serum to clearly distinguish between D- and L-glutamic acids and the various dipeptides of glutamic acid, as well as the somewhat surprising effectiveness of the branched tripeptide, α,γ-L-Glu-(D-Glu)$_2$, remain unresolved problems. Possibly, the specificity of the antibody is directed against a conformation which is dependent on secondary and tertiary structural features. The demonstration of such dependence must await the availability of larger peptide inhibitors. It has been postulated that the specific interaction rests heavily upon the distance between repeating carboxyl groups in the polypeptide (Bruckner *et al.*, 1958). While the data obtained with the copolymers of glutamic acid and glycine or alanine do not conflict with such a hypothesis, the results may also be interpreted in other ways. Alternatively, the complete absence of L-glutamic acid from the anthrax polypeptide cannot be established. Should a small proportion of the glutamic acid in the polypeptide be of the L configuration, the specificity of the antibody could involve such residues.

In recent years a number of reports concerning the extent of the determinant groups on polypeptide antigens and polypeptide–protein conjugates have appeared. Sage *et al.* (1964) found that the reaction between rabbit antipoly-L-alanine–protein conjugate and the homologous antigen was inhibited maximally by pentaalanine when the dipeptide to the hexapeptide were tested. The average length of the peptide chains attached to the protein was 7.6 residues. The hexapeptide was actually less active than the pentapeptide in these studies, an effect that may be due to change in

tertiary structure. Somewhat similar findings were noted by Kabat for the inhibition of an α-1,4-dextran–antidextran system by maltose oligosaccharides (Kabat, 1954). Antibodies to polylysyl rabbit serum albumin have been obtained in rabbits and the homologous precipitin reaction was inhibited by oligolysines of increasing chain length (Arnon *et al.*, 1965b). Inhibiting efficiency rose steeply up to pentalysine and then more gradually up to nonalysine. Since the average chain length of the lysine peptides attached to rabbit albumin was 5.5 residues, it was not possible to conclude that this represented the maximum size of the combining region on the antigen. The increased effectiveness of peptides up to nonalysine could have been due either to the presentation of more combinations of five residues or to the presence of some antibody molecules with larger combining sites. In the former case, one might expect to see further gains with even larger peptides, particularly since the increment between octalysine and nonalysine was about as great as that between hexalysine and octalysine. However, decalysine was indistinguishable from nonalysine in the inhibition tests.

Investigations with natural and synthetic polymers containing more than one kind of amino acid have also yielded useful, though necessarily more limited, information about the size of the determinant group. Maurer (1964) obtained antisera against copolymers of glutamic acid, alanine, and either tyrosine or lysine and found that while di- and tripeptides of glutamic acid were ineffective inhibitors of the homologous precipitin reactions, the penta- and hexapeptides were quite potent. Cebra (1961) found that he required octapeptides from a partial hydrolysate of silk fibroin to significantly inhibit the reaction between undegraded silk fibroin and its homologous antibody. These studies suffer from the relative complexity of the antigens and consequent lack of information concerning the compositions of the determinant groups. However, disregarding Cebra's findings, which contain the highest degree of uncertainty, the conclusions regarding determinant group size reached by the above investigators agree reasonably well with those of Kabat (1958) for the dextran–antidextran system.

The anthrax polypeptide–antipolypeptide system is limited neither by the short peptide chains of the conjugated antigens nor by the complexity of the copolymers and, thus, represents the closest polypeptide analogy to the dextran system that has yet been studied. While inhibition results with heterogeneous commercial preparations of the tetrapeptide and hexapeptide of D-glutamic acid suggest that peptides larger than the homogeneous ones thus far investigated will give increased efficiency (Figure 2), delineation of the extent of the determinant group on this antigen must await the availability of homogeneous preparations of larger peptides. Investigations of this kind are currently in progress.

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Immunochemical Studies on the Poly-γ-D-glutamyl Capsule of *Bacillus anthracis*. II. The Synthesis of Eight Dipeptides and Four Tripeptides of Glutamic Acid*

Danute E. Nitecki and Joel W. Goodman

ABSTRACT: Eight dipeptides and four branched tripeptides of D- and L-glutamic acid were synthesized to be used as hapten inhibitors in the homologous immune system of capsular poly-γ-D-glutamic acid isolated from *Bacillus anthracis*. *N*-Carbobenzoxy and benzyl esters were used as protective groups in the synthesis, and the coupling was achieved with a dicyclohexylcarbodiimide reagent. Protective groups were removed by catalytic hydrogenolysis in one step. The purity of the starting materials and products in each synthetic step was ascertained by thin layer chromatography on silica gel, paper chromatography, and high voltage electrophoresis. The free peptides and their hydrolysates reacted quantitatively with ninhydrin and 2,4-dinitrobenzene-1-sulfonic acid reagents and the peptide hydrolysates showed the expected specific optical rotations. Diastereomeric α-dipeptides and tripeptides were shown to be separable by paper chromatography in several solvents and by high voltage electrophoresis.

Information about the extent of the areas of antigen molecules involved in combining with antibodies (determinant groups) as well as the contributions of various structural features to specificity can be obtained by hapten inhibition of the precipitin reaction.

In this investigation, the antigen studied was a capsular polypeptide isolated from a strain of *Bacillus anthracis*, to which rabbit antisera have been obtained. The polypeptide was shown to be essentially pure poly-γ-D-glutamic acid (Goodman and Nitecki, 1966), an antigen of relatively simple structure. The serological specificity of its reaction with rabbit antisera and hapten inhibition of the homologous immune system using amino acids and synthetic di- and tripeptides of glutamic acid were investigated. The results of serological investigation are presented in another publication (Goodman and Nitecki, 1966).

For this purpose eight dipeptides and four tripeptides of glutamic acid were synthesized. There are four possible α-dipeptides (**1–4**)[1] and four possible γ-dipeptides (**5–8**). Four of the dipeptides have been synthesized before by a different method (Sachs and Brand, 1953a).

In addition, four branched tripeptides have been synthesized in which one glutamic acid moiety is substituted in both α and γ positions by glutamic acid units (**9–12**). In two of these, the central α,γ-substituted glutamic acid is of opposite configuration to the other two glutamic acid moieties (**9** and **10**); the other two

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[1] The abbreviations used are described by Young (1962).