Synthesis of Poly-γ-glutamyl-γ-aminobutyric Acids and Their Reactivity with Antiserum against *Bacillus anthracis* Polypeptide†

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ABSTRACT: To test the importance of the carboxyl group in native polyglutamic acid in the precipitation reaction with immune serum against anthrax, poly-γ-glutamyl-γ-aminobutyric acids in the D and L series were synthesized. This polymer is similar to anthrax polypeptide, except every second carboxyl group is missing. The α-*tert*-butylglutamyl-γ-aminobutyric acid pentachlorophenyl esters, D and L, were polymerized to give the corresponding poly-α-*tert*-butylglutamyl-γ-aminobutyric acids. After removal of the *tert*-butyl protecting groups, poly-γ-L-glutamyl-γ-aminobutyric acid (PLGA) and poly-γ-D-glutamyl-γ-aminobutyric acid (PDGA) were obtained with molecular weights of 12,500 and 12,300, respectively. Both PLGA and PDGA reacted with about 35% of the antibody specific for the poly-γ-D-glutamic acid capsular material of *Bacillus anthracis;* however, PLGA can precipitate this entire fraction, while PDGA can precipitate only about half of it.

The capsular substance of *Bacillus anthracis* (Ivanovics and Bruckner, 1937) has been shown to consist entirely of γ-glutamyl residues of the D configuration (Kovacs and Bruckner, 1952; Kovacs *et al.*, 1953; Bruckner *et al.*, 1953a; Bruckner *et al.*, 1953b; Bruckner *et al.*, 1958). Synthetic poly-γ-D-glutamic acid and native polyglutamic acid gave the same results in precipitin tests with anthrax antiserum (Bruckner *et al.*, 1958). In addition, L,D mesoid poly-γ-glutamic acid (Bruckner *et al.*, 1957; Bruckner *et al.*, 1955) gave a weaker precipitin reaction with anthrax immune serum (Bruckner and Kovacs, 1957). In order to explain the reactivity of the L,D mesoid poly-γ-glutamic acid, it was postulated that the distance between the α-carboxyl groups on the asymmetric carbons with D configuration played an important role in the precipitation reaction (Kovacs, 1962). To investigate this problem further as well as to obtain data on the role of the carboxyl groups in the serological reaction, a series of sequential polypeptides were synthesized with glycyl, β-alanyl, and γ-aminobutyryl residues separating the γ-glutamyl residues with a gradual increase in the distance of carboxyl groups. The synthesis of the L,D isomers of the first two members of this series, *i.e.*, poly-γ-glutamylglycine and poly-γ-glutamyl-β-alanine were previously reported (Kovacs *et al.*, 1968; Kovacs and Johnson, 1965). The serological reactions with antianthrax immune serum were also reported; this investigation showed that neither the D nor the L isomer of the above two polypeptides gave a precipitation reaction. However, the D isomer inhibited the precipitation reaction between anthrax polypeptide and its antiserum to a greater extent than the L isomer (Goodman and Nitecki, 1966).

This paper reports the synthesis of poly-γ-D- and L-glutamyl-γ-amino butyric acid together with its serological reaction with anthrax antiserum.

The D isomer of poly-γ-glutamyl-γ-aminobutyric acid was expected to give precipitation reactions with anthrax antiserum based on the considerations discussed below. Figure 1 shows the molecular model (Stuart and Briegleb) of a short segment of poly-γ-D- and L-glutamic acids, which was constructed taking into consideration the planarity of the trans peptide bond and the stability of the staggered conformation. The model shows that every second carboxyl group is on the same side of the peptide chain and the second and fourth are on the opposite side. Under the conditions used for precipitation reaction with anthrax antiserum, namely at pH 7.4, the carboxyl groups are ionized. The negative charge on the carboxyl groups would tend to cause their maximum separation. The conformation shown by Figure 1 is strengthened by the electrostatic repulsion between the ionized carboxyl groups since this arrangement also represents the maximum separation of the negative charges. Entropy effects rule out straight-chain structure for a long segment in the molecule; however, it is likely that there are portions of the peptide chain with this conformation. It was shown by Edelhoch (Edelhoch and Bateman, 1957; Edelhoch and Lippoldt, 1960) that native polyglutamic acid, when ionized, is in an expanded state. Recent investigation of the secondary structure of poly-γ-glutamic acid by Balasubramanian *et al.*[1] seems to indicate that at high pH values it may be a disordered coil.

Present study of the secondary structure of PDGA using optical rotatory dispersion (ORD) and circular dichroism (CD) seems to indicate that this sequential polypeptide is very similar to native polyglutamic acid. Figure 2 indicates the configuration of a short segment of poly-γ-D- and L-glutamyl-γ-aminobutyric acids. Distances between the repeating carboxyl groups with D configuration of the α-carbon atoms are the same as the first and third carboxyl groups on the peptide chain of the native polyglutamic acid; in other words, every second carboxyl group is missing. L,D Mesoid poly-γ-glutamic acid also gives precipitin reaction with anthrax immune serum; in this polypeptide every second car-

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[1] D. Balasubramanian, C. Kalita, and J. Kovacs, manuscript in preparation.

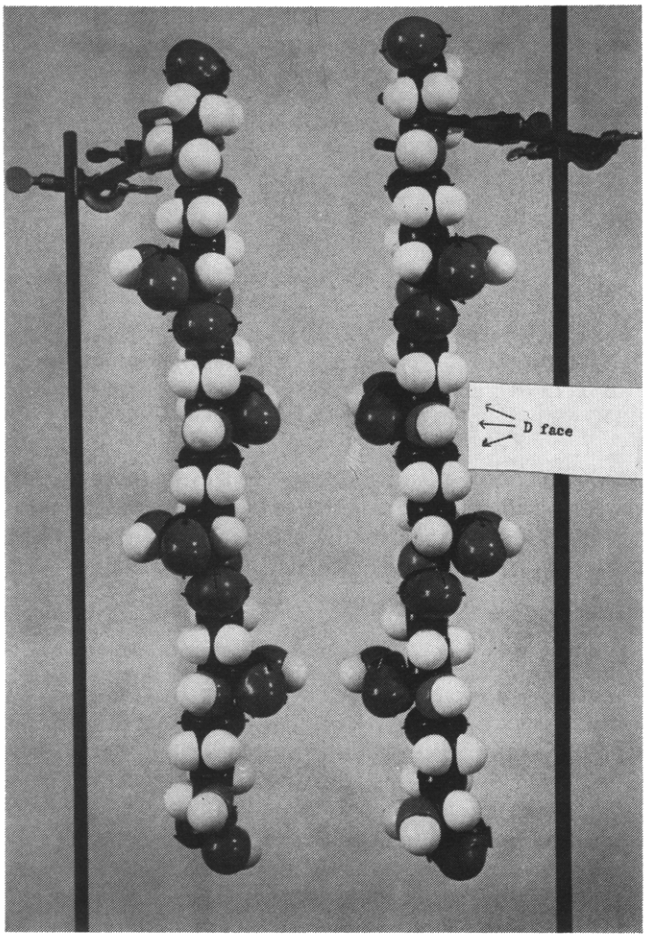


FIGURE 1: Poly-γ-L-glutamic acid and poly-γ-D-glutamic acid.

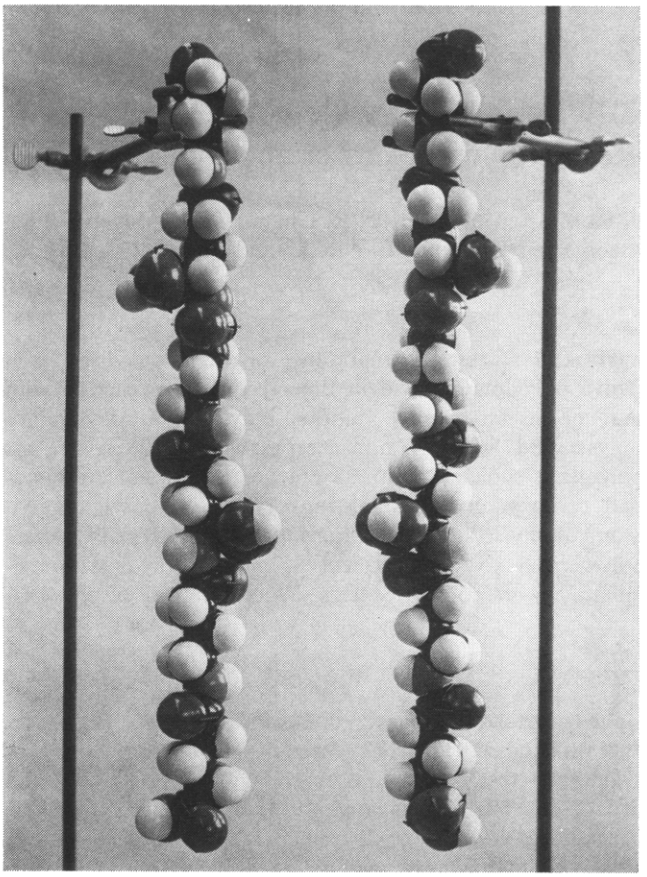


FIGURE 2: Poly-γ-L-glutamyl-γ-aminobutyric acid and poly-γ-D-glutamyl-γ-aminobutyric acid.

boxyl group is on an L-glutamyl residue. Poly-γ-D-glutamyl-γ-aminobutyric acid possesses the same peptide chain as poly-γ-D-glutamic acid except that every other carboxyl group is missing as indicated by Figure 2. The scheme below indicates the synthetic route.

$$\underset{\text{I}}{C_6H_5CH_2OCONHCH(CO_2Bu^t)CH_2CH_2CO_2H} + \underset{\text{II}}{HCl\cdot H_2N(CH_2)_3CO_2R} \xrightarrow[\text{anhyd}]{\text{mixed}} \text{III}$$

$$C_6H_5CH_2OCONHCH(CO_2Bu^t)CH_2CH_2CONHCH_2CH_2CH_2CO_2R$$

III, R = Me; IV, R = H; V, R = C_6Cl_5

$$HCl\cdot H_2NCHCO_2Bu^t$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$CONHCH_2CH_2CH_2CO_2C_6Cl_5$$

VI

$$\left[\begin{array}{l} \ldots NHCHCO_2R \\ \quad | \\ \quad CH_2 \\ \quad | \\ \quad CH_2 \\ \quad | \\ \quad CONHCH_2CH_2CH_2CO\ldots \end{array}\right]_n$$

VII, R = *tert*-Bu; VIII, R = H

The dipeptide methyl ester III was prepared by coupling *N*-benzyloxycarbonyl-α-*tert*-butylglutamic acid (I) (Kovacs *et al.*, 1968) with methyl-γ-aminobutyrate (II) through mixed anhydride coupling. The resulting *N*-benzyloxycarbonyl-α-*tert*-butylglutamyl-γ-aminobutyric acid methyl ester (III) was saponified (4 min) to the dipeptide free acid IV, which was characterized through its dicylcohexylamine salt. It was observed that prolonged hydrolysis yielded undesired side reaction, *i.e.*, the removal of the *tert*-butyl ester groups from III. A similar anomalous reaction was observed for *N*-benzyloxycarbonyl-β-*tert*-butyl-L-aspartic acid containing pentapeptide ethyl ester (Bajusz *et al.*, 1964). The dipeptide pentachlorophenyl ester V was prepared through the "reverse" dicyclohexlcarbodiimide procedure (Kovacs *et al.*, 1967, 1969). *N*-Benzyloxycarbonyl-α-*tert*-butylglutamyl-γ-aminobutyric acid pentachlorophenyl ester (V) was subjected to catalytic hydrogenation to give α-*tert*-butylglutamyl-γ-aminobutyric acid pentachlorophenyl ester hydrochloride (VI).

Polymerization was carried out starting with a highly concentrated solution of the hydrochloride salt VI in dimethylformamide in the presence of 2–2.5 equiv of triethylamine Kovacs *et al.*, 1968). Extreme care was taken to use pure active ester hydrochloride VI as well as purified solvent and triethylamine to avoid undesired side reactions which would lower the degree of polymerization. The crude polymer was washed extensively with methanol to remove cyclopeptides and low molecular weight polymers. The resulting poly-α-*tert*-butylglutamyl-γ-aminobutyric acid (VII) exhibited no active ester peak at 5.6 μ.

The *tert*-butyl groups were cleaved from VII using 90% trifluoroacetic acid to give poly-γ-glutamyl-γ-aminobutyric acid (VIII). Removal of the *tert*-butyl group from *N*-carbobenzoxy-α-*tert*-butyl-γ-D-glutamyl-β-alanine under these

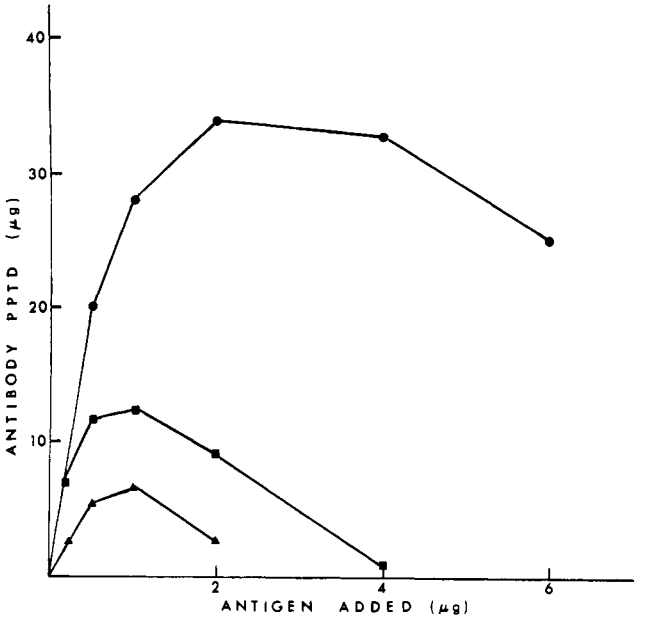


FIGURE 3: Precipitin reactions between 75 μl of antiserum against poly-γ-D-Glu and ● poly-γ-D-Glu, ■ PLGA, and ▲ PDGA.

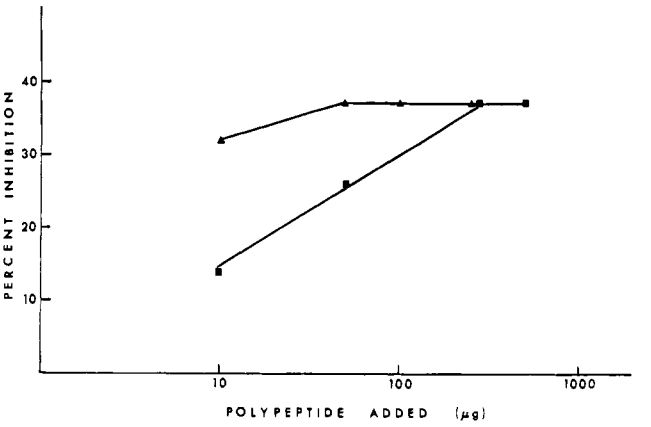


FIGURE 4: Inhibition of the precipitin reaction between 75 μl of antiserum and 2 μg of poly-γ-D-Glu by ■ PLGA and ▲ PDGA.

conditions did not yield any transpeptidized product (Kovacs and Johnson, 1965). Therefore it was assumed that this polymer is also free of transpeptidation.

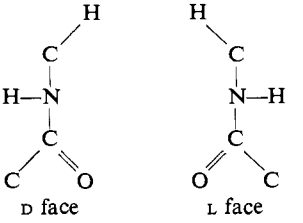
The weight-average molecular weights of the polymers were determined by ultracentrifugation using the equilibrium method described by Schachman (Schachman, 1959). Poly-γ-D- and L-glutamyl-γ-aminobutyric acids (VIII) had weight-average molecular weights of 12,300 and 12,500, respectively.

Serological Testing. The D and L polymers were assayed for reactivity with rabbit antiserum specific for the poly-γ-D-glutamic acid capsular material of *Bacillus anthracis*. The properties of the antiserum and the polypeptide, as well as the immunologic methods, have been described (Goodman and Nitecki, 1966; Goodman *et al.*, 1968). For these experiments, 75 μl of serum containing 450 μg of antibody/ml were mixed with varying quantities of polypeptide, individually or in combination, in 3.0-ml conical centrifuge tubes. Solutions of the polypeptides were adjusted to pH 7.3–7.7 prior to use. Final volumes were adjusted to 0.7 ml with saline. The tubes were refrigerated for 5 days and then centrifuged and washed three times with 0.5 ml of saline. The washed precipitates were dried *in vacuo* over phosphorus pentoxide and analyzed for protein colorimetrically, using a modification of the Folin–Ciocalteu method in which the analyses were done directly in the centrifuge tubes in a total volume of 1.0 ml (Goodman *et al.*, 1968). The results of quantitative precipitin tests are shown in Figure 3. Poly-γ-D-Glu precipitated 450 μg/ml of serum. PLGA precipitated 150 μg/ml or 34% of this antibody whereas PDGA precipitated only 85 μg or 19%. When the alternating polymers were used to inhibit the homologous precipitin reaction, they each inhibited to the extent of 35%, although less PDGA than PLGA was required to reach this limit (Figure 4). Thus, each of the polymers reacts with about 35% of the antibody, but PLGA can precipitate this entire fraction while PDGA can precipitate only about half of it. The basis for this difference is unclear.

It was established that PDGA and PLGA react with the same fraction of antibody by doing precipitin tests with each in the presence of a large excess of the other. Under these circumstances, no precipitation was observed due to the large excess of antigen. Thus each of the polymers inhibited precipitation by the other, which showed that they reacted with the same 35% fraction of antibody. This was confirmed by inhibiting the homologous precipitin reaction with mixtures of PLGA and PDGA. The maximum inhibition obtained was 35%. If the two reacted with distinct fractions of antibody, a maximum inhibition of 70% would have been anticipated. In addition, after removal of the antibody precipitated by PLGA, precipitation by the homologous antigen could not be inhibited by either PLGA or PDGA.

These results were somewhat unexpected in that PDGA proved to be a relatively poor ligand for the antibody and PLGA reacted with as large a fraction of antibody as did PDGA. The results indicated that the specificity of about two-thirds of the antibody in this serum is directed against structural features of the antigen which require carboxyl groups on adjacent residues. In conjunction with previous studies which had shown that poly-γ-D-glutamylglycine and poly-γ-D-glutamyl-β-alanine reacted with more than 50% of the antibody in the serum (Goodman and Nitecki, 1966), it may be inferred that the spacing of adjacent carboxyl groups is critical for reactivity with antibody.

It was not expected that PLGA will give precipitation reaction with anthrax immune serum based on the results of previously tested polypeptides. After the serological reaction was completed, a study of the model of PLGA and PDGA seems to offer the following hypothesis. Both PDGA and PLGA have the same "faces" at the amide bond of the amino group of the γ-aminobutyric acid and γ-carboxyl group of the glutamic acid; in comparison, anthrax polypeptide has only D faces, and poly-γ-L-glutamic acid has only L faces, at each amine bond. This situation can be compared with the enzymatic reduction of acetaldehyde; this symmetrical molecule has enantiomeric faces and only one side fits to the active site of NHD enzyme.



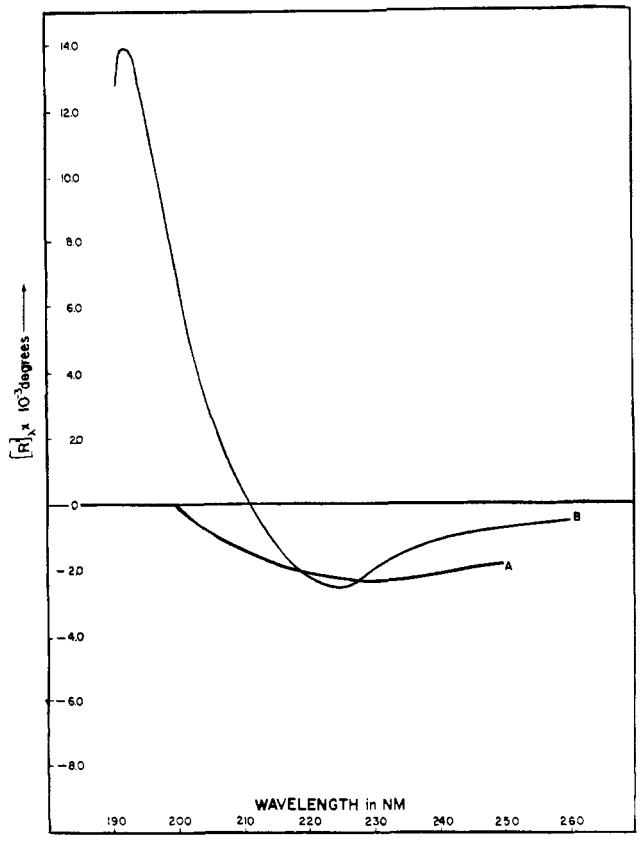


FIGURE 5: ORD of poly-γ-D-glutamyl-γ-aminobutyric acid (PDGA): (A) at pH 9.0 and (B) at pH 2.0.

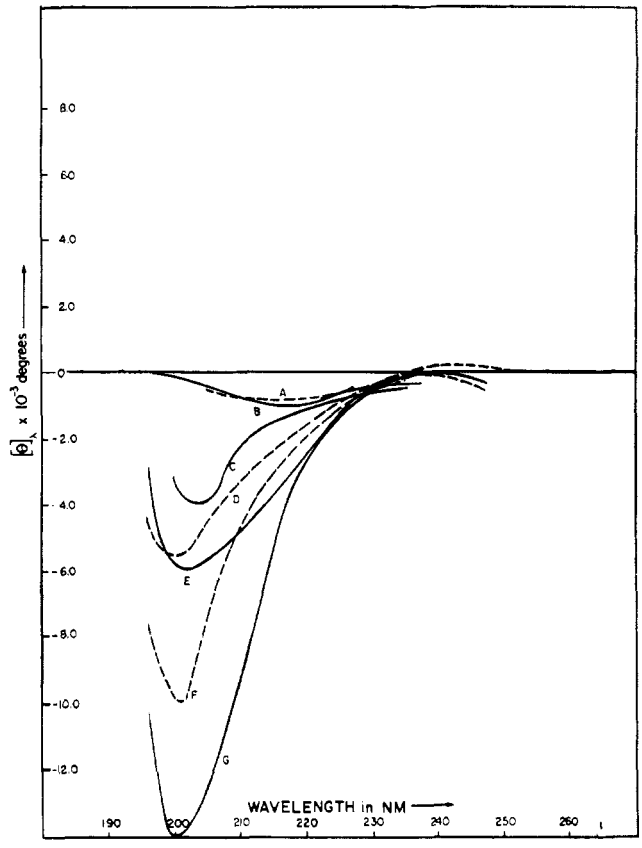


FIGURE 6: CD of poly-γ-D-glutamyl-γ-aminobutyric acid (PDGA): (A) at pH 9.0, (B) at pH 7.3, (C) at pH 6.0, (D) at pH 5.0, (E) at pH 4.0, (F) at pH 3.0, (G) at pH 2.0.

If the amide group is important for the serological reaction in addition to carboxyl and other groups, then both PDGA and PLGA expected to react to a certain extent with the antibody against anthrax, since these groups are spaced the same way as in poly-γ-D-glutamic acid. To test this hypothesis poly-γ-glutamyl-γ-aminobutyric acid derivatives will be prepared for testing.

ORD and CD Spectra of Poly-γ-D-glutamyl-γ-aminobutyric Acid. Figure 5 shows the Cotton effects of PDGA in aqueous solution. The rotatory dispersion of the ionzied form of PDGA at pH 9 is characterized by a negative Cotton effect at 230 nm with a rotational amplitude of −2371° and a crossover point at 197 nm. At lower wavelengths the curve gets unreliable owing to low rotations, high absorption, and noise. In the un-ionized form of PDGA (pH 2) the ORD peak is positioned at 225 nm with an amplitude of −2538°, the crossover point is at 212 nm, and there is a large positive Cotton effect at 193 nm with a peak height of 14,091°.

Circular dichroism spectra of poly-γ-D-glutamyl-γ-aminobutyric acid at various degrees of ionization are shown in Figure 6. At high degrees of dissociation the CD spectra are characterized by small negative Cotton effects. At pH 9 the trough is at 215 nm with a rotational amplitude of −925°. In constrast the un-ionized form of the polypeptide at pH 2 exhibits an intense negative Cotton effect with an amplitude of −13,951° at 200 nm. From Figure 6 it can be seen that as the pH is progressively changed from 2 to 9, there is a reduction in the amplitude of negative Cotton effect as well as a redshift of the trough. However, the overall qualitative profile of the spectra is not altered by variation of the pH from 2 to 9.

The spectral feature of PDGA described above closely resembles that of poly-γ-D-glutamic acid, which does not exhibit acid from *B. subtilis* and *B. anthracis* and synthetic poly-γ-L-glutamic conformational order. To the extent that CD spectrum of a polypeptide is characteristic and diagnostic of chain conformations, it is suggested that at high pH PDGA may be a disordered coil.

Experimental Section

All melting points are uncorrected. The microanalyses were carried out either by Dr. F. B. Strauss, Oxford, England, or by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. Infrared spectra were determined in potassium bromide pellets using a Beckman Model IR-8 spectrophotometer. Thin-layer chromatography were carried out on precoated silica gel analytical plates F254 (Brinkman); unless otherwise stated spots were located by ultraviolet light. Optical rotations were taken on a Rudolph spectropolarimeter Model 200S-340-80Q3. Weight-average molecular weights were determined in the Spinco Model E analytical untracentrifuge by the sedimentation equilibrium method.

N-Benzyloxycarbonyl-α-tert-butyl-L-glutamyl-γ-aminobutyric Acid Methyl Ester (III). *N*-Methylmorpholine (2.4 ml, 22.9 mmoles) and isobutyl chloroformate (3.1 ml, 23.6 mmoles) in ethyl acetate (80 ml) at −15° were added to a solution of *N*-benzloxycarbonyl-α-*tert*-butyl-L-glutamic acid (7.6 g, 22.6 mmoles) in ethyl acetate (40 ml) at −15°. After 10 min, γ-aminobutyric acid methyl ester hydrochloride (3.5 g, 22.9 mmoles) and triethylamine (3.2 ml, 22.9 mmoles)

were added and the mixture was kept at −15° for 16 hr and at room temperature for 2 hr. The reaction mixture was diluted with ethyl acetate (200 ml), washed consecutively with distilled water, 2% aqueous sodium bicarbonate, 1 N hydrochloric acid, distilled water, dried, and concentrated under reduced pressure. The oily residue was crystallized from ethyl acetate–pentane to give the dipeptide methyl ester III (7.0 g, 70%): mp 58–60°; $[\alpha]^{25}D$ −16.5° (*c* 2.1, ethanol), λ_{max} 5.72 (methyl ester), 5.78 (*tert*-butyl ester), 6.05 (amide I), 6.6 μ (amide II). Found: C, 60.5; H, 7.2; N, 6.1. $C_{22}H_{32}N_2O_7$ requires C, 60.5; H, 7.4; N, 6.4%.

*N-Benzyloxycarbonyl-α-tert-butyl-*D*-glutamyl-γ-aminobutyric Acid Methyl Ester (III).* This compound was prepared in a similar manner to the L isomer in 56% yield: mp 58–60°; $[\alpha]^{22}D$ 16.54° (*c* 1.97, ethanol) and 15.6° (*c* 1.16, methanol). Found: C, 60.3; H, 7.4; N, 6.6%.

*N-Benzyloxycarbonyl-α-tert-butyl-*D*-glutamyl-γ-aminobutyric Acid (IV).* *N*-Benzyloxycarbonyl-α-*tert*-butyl-D-glutamyl-γ-aminobutyric acid methyl ester (2.1 g, 4.8 mmoles) was dissolved in dioxane (2.7 ml) with gentle warming. To this was added 1 N sodium hydroxide (7.2 ml) forming a cloudy solution which cleared up in 4 min. An equimolar amount of 1 N hydrochloric acid (7.2 ml) was added and the reaction mixture was immediately extracted with ethyl acetate (40 ml). The mother liquor was saturated with sodium chloride and additional product was extracted with ethyl acetate (10 ml). The combined organic layers were washed three times with water (50 ml) and dried. The solvent was removed under reduced pressure to yield an oil (1.9 g, 95%). Tlc (1-butanol–acetic acid–water, 4:1:1) showed one spot. The L isomer of IV was prepared similarly.

When the saponification of methyl ester III was carried out in acetone for 40 min a mixture of IV and *N*-benzyloxycarbonyl-D-glutamyl-γ-aminobutyric acid was obtained as indicated by tlc. The oily mixture (4 g) was dissolved in ether (50 ml) and upon addition of dicyclohexylamine (10 ml) crystalline material separated, which was filtered and recrystallized from methanol–ether (1:4): yield 2.3 g; mp 153–155°; $[\alpha]^{22}D$ −5.2° (*c* 1.04, methanol). The elemental analysis indicated that this is *N-benzyloxycarbonyl-*D*-glutamyl-γ-aminobutyric acid didicyclohexylamine salt.* Found: C, 67.4; H, 9.4; N, 7.6. $C_{41}H_{68}N_4O_7$ requires C, 67.6; H, 9.4; N, 7.7%. From the mother liquors 1.4 g of dicyclohexylamine salt of IV was isolated: mp 113–117°; $[\alpha]^{22}D$ 7.1° (*c* 1.06, methanol). Found: C, 65.8; H, 8.8; N, 7.1. $C_{33}H_{53}N_3O_7$ requires C, 65.6, H, 8.9; N, 7.0%.

*N-Benzyloxycarbonyl-α-tert-butyl-*L*-glutamyl-γ-aminobutyric Acid Pentachlorophenyl Ester (V).* To a solution of pentachlorophenol (1.8 g, 6.8 mmoles) and dicyclohexylcarbodiimide (1.34 g, 6.8 mmoles) in ethyl acetate (110 ml), the dipeptide free acid IV (2.8 g, 6.8 mmoles) in ethyl acetate (30 ml) was added at −15°. The mixture was stirred at −15° overnight, the dicyclohexylurea was filtered, and the filtrate was evaporated under reduced pressure. The resulting solid was dissolved in a minimum amount of hot ethyl acetate, kept at 0° for 3 hr, and additional dicyclohexylurea filtered. The filtrate was again evaporated and the solid residue crystallized twice from methanol to afford the dipeptide pentachlorophenyl ester V (1.97 g, 45%): mp 140.5–142°; $[\alpha]^{23}D$ −10.0° (*c* 1.0, dimethylformamide); λ_{max} 5.62 (pentachlorophenyl ester), 5.78 (*tert*-butyl ester), 5.90 (benzyloxycarbonyl), 6.05 (amide I), 6.48 μ (amide II). Tlc (ethyl acetate–methanol, 19:1 and benzene–methanol, 1:1) showed one spot. Found: C, 48.2; H, 4.4; N, 4.3; Cl, 26.4. $C_{27}H_{29}N_2O_7Cl_5$ requires C, 48.3; H, 4.4; N, 4.2; Cl, 26.4%.

*N-Benzyloxycarbonyl-α-tert-butyl-*D*-glutamyl-γ-aminobutyric Acid Pentachlorophenyl Ester (V).* This ester was prepared in a similar manner to the L isomer in 55% yield: mp 141–142°; $[\alpha]^{25}D$ 10° (*c* 0.89, dimethylformamide), tlc (ethyl acetate–methanol, 19:1 and benzene–methanol, 1:1) showed 1 spot. Found: C, 48.2; H, 4.4; N, 4.3; Cl 26.4%.

*α-tert-Butyl-*D*-glutamyl-γ-aminobutyric Acid Pentachlorophenyl Ester Hydrochloride (VI).* A suspension of 10% palladium on charcoal (175 mg) in glacial acetic acid (0.61 ml) and absolute methanol (10 ml) was activated with dry hydrogen. The solvent was decanted and the catalyst washed twice with absolute methanol (10 ml). Methanolic hydrogen chloride (0.64 ml, 1.6 mmoles) and a solution of *N*-benzyloxycarbonyl-α-*tert*-butyl-D-glutamyl-γ-aminobutyric acid pentachlorophenyl ester (1.1 g, 1.6 mmoles) in a 5:2 mixture of tetrahydrofuran–methanol (14 ml) were added. Hydrogenation was completed in 10 min, the catalyst was filtered, the filtrate was concentrated under reduced pressure, and the oily residue was crystallized from anhydrous methanol–ether. After filtration it was washed with methanol–ether (1:10) and dried: 790 mg; 84%; mp 156–156.5° dec; $[\alpha]^{26}D$ −8.8° (*c* 0.55, methanol); tlc (dichloromethane–methanol, 9:1 and ethyl acetate–methanol, 9:1) showed 1 spot; λ_{max} 3.4 broad (NH_3^+), 5.6 (pentachlorophenyl ester), 5.79 (*tert*-butyl ester), 6.05 (amide I), 6.5 μ (amide II); the benzyloxycarbonyl peak was absent. A sample was recrystallized from methanol–ether: mp 156–157.5° dec; $[\alpha]^{22}D$ −9.0° (*c* 2, methanol). Found: C, 40.1; H, 4.4; N, 5.2; Cl, 37.4. $C_{19}H_{24}N_2O_5Cl_6$ requires C, 39.8; H, 4.2; N, 5.0; Cl, 37.1%.

*α-tert-Butyl-*L*-glutamyl-γ-aminobutyric Acid Pentachlorophenyl Ester Hydrochloride (VI).* It was prepared in a similar manner to the D isomer in 60% yield; mp 155–156° dec; $[\alpha]^{23}D$ 9.0° (*c* 3.1, methanol). The ir spectrum was identical with that of the D isomer. Found: C, 40.1; H, 4.4; N, 5.2; Cl, 37.4%.

*N-Acetyl-γ-*D*-glutamyl-γ-aminobutyric Acid Methyl Ester.* *N*-Benzyloxycarbonyl-α-*tert*-butyl-D-glutamyl-γ-aminobutyric acid methyl ester (1.31 g, 3 mmoles) in methanol (50 ml) was hydrogenated in the presence of methanolic hydrogen chloride (0.68 ml, 3 mmoles) in the usual manner. The resulting oil was precipitated from methanol–ether to give α-*tert*-butyl-γ-D-glutamyl-γ-aminobutyric acid (1 g, 99%). The product, which could not be crystallized, was used for the next step.

To α-*tert*-butyl-γ-D-glutamyl-γ-aminobutyric acid pentachlorophenyl ester hydrochloride (1.01 g, 3 mmoles) in dichloromethane (10 ml), triethylamine (0.42 ml, 3.03 mmoles), and acetic anhydride (0.29 ml, 3.03 mmoles) were added and the mixture was stirred overnight. The mixture was diluted with dichloromethane and washed consecutively with distilled water, 2.5% sodium bicarbonate, distilled water, 1 N hydrochloric acid, and distilled water. The solvent was dried and concentrated under reduced pressure. The resulting oil was used for the next step.

N-Acetyl-α-*tert*-butyl-γ-D-glutamyl-γ-aminobutyric acid methyl ester (823 mg, 2.4 mmoles) was treated with 90% trifluoroacetic acid (16 ml) in the usual manner. The resulting oil, which did not crystallize, was lyophilized to give *N*-acetyl-γ-D-glutamyl-γ-aminobutyric acid (150 mg, 22%). Tlc (butanol–acetic acid–water, 4:1:1) indicated a pure material. Found C, 49.6; H, 6.7; N, 9.0. $C_{12}H_{20}N_2O_6$ requires C,50.0; H, 7.0; N, 9.7%.

*Poly-α-tert-butyl-γ-*L*-glutamyl-γ-aminobutyric Acid (VII).* Triethylamine (0.46 ml, 3.29 mmoles) was added to α-*tert*-butyl-γ-L-glutamyl-γ-aminobutyric acid pentachlorophenyl

ester hydrochloride (0.93 g, 1.63 mmoles) in dimethylformamide (1.1 ml). This mixture was shaken vigorously for 2 days. After trituration with ether the solid was filtered and washed several times with methanol, distilled water, methanol, and ether to give the pure protected polymer: 0.2 g; 45%; λ_{max} 5.76 (*tert*-butyl ester), 6.05 (amide I), 6.48 μ (amide II); pentachlorophenyl ester peak was absent. Found: C, 57.6; H, 8.2; N, 10.5. $C_{13}H_{22}N_2O_4$ requires C, 57.8; H, 8.2; N, 10.4%.

*Poly-α-tert-butyl-γ-*D*-glutamyl-γ-aminobutyric Acid (VII).* This polymer was prepared as described above for the L isomer (96 mg, 41%). Found: C, 57.5; H, 8.3; N, 10.3%.

*Poly-γ-*L*-glutamyl-γ-aminobutyric Acid (VIII).* Poly-α-*tert*-butyl-γ-L-glutamyl-γ-aminobutyric acid (0.10 g, 0.37 mmole) was dissolved in 90% aqueous trifluoroacetic acid (3 ml) and kept at room temperature for 50 min. Ether (200 ml) was added and the resulting white precipitate was collected by centrifugation. The precipitate was washed three times with ether and the polymer was dried over phosphorus pentoxide at 78° under reduced pressure, yielding 49 mg; 62%; λ_{max} 5.8 (COOH), 6.04 (amide I), and 6.45 μ (amide II). Found: C, 49.8; H, 6.7; N, 13.1. $C_9H_{14}N_2O_4$ requires C, 50.5; H, 6.6; N, 13.1%.

*Poly-γ-*D*-glutamyl-γ-aminobutyric acid (VIII)* was prepared as described above for the L isomer (64%). Found: C, 50.3; H, 6.6; N, 13.4%.

*Optical Purity of Poly-γ-*L*-glutamyl-γ-aminobutyric Acid.* Poly-γ-L-glutamyl-γ-aminobutyric acid (40 mg, 0.187 mmole) in 6 N hydrochloric acid (1.0 ml) and glacial acetic acid (1.0 ml) was refluxed for 24 hr. The solution was diluted to a volume of 3 ml with 6 N hydrochloric acid. The observed optical rotation of this solution was $\alpha^{23}D$ 0.36° ± 0.01°.

A control experiment was carried out with L-glutamic acid (27.5 mg, 0.187 mmole) and γ-aminobutyric acid (19.3 mg, 0.187 mmole) using the same procedure described above to give $\alpha^{22}D$ 0.35° ± 0.01°. This would indicate 100% optical purity.

The optical purity of the D polymer was determined as described for the L isomer and it was found to be 99% ± 1% optically pure.

Weight-Average Molecular Weight. The weight-average molecular weight was determined by sedimentation equilibrium (Schachman, 1959) at 23° in phosphate buffer at pH 7.3, at a concentration range of 3.7–5.55 mg/ml and rotor speed of 18,000 at a schlieren angle of 65°. The phosphate buffer of pH 7.3, which contains 50 ml of 0.1 M KH_2PO_4 and 37.0 ml of 0.1 M NaOH, was diluted to 100 ml with water. The polymers showed a single peak and from the equilibrium sedimentation data $\bar{M}_w$ of 12,500 for the L isomer and 12,300 for the D isomer were calculated assuming a partial specific volume of 0.72.

ORD and CD Studies. Optical rotatory dispersion and circular dichroism measurements were made with the Cary 60 automatic recording spectropolarimeter. The cell paths used were between 0.1 and 5 mm and the concentration was 10 mg of polymer per 100 ml of water. The pH of the solution was obtained by adding the necessary amount of sulfuric acid solution or sodium hydroxide solution.

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