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Synthesis and Conformational Studies of Poly-β-(γ-glutamyl)-aspartic Acid

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High molecular-weight ($\bar{M}$W 28,000) water-soluble poly-β-(γ-glutamyl)-aspartic acid (X) was synthesized in both L- and D- series through the polymerization of α-*t*-butylglutamyl-α-*t*-butylaspartic acid pentachlorophenyl ester hydrochloride (VI), followed by the removal of the carboxyl protecting group. The ORD and CD spectra of X are reported. This polymer is a model to test the structural requirements for the precipitation reaction with antianthrax immune serum.

The structure of native polyglutamic acid has been shown to be poly-γ-D-glutamic acid (*1–5*) by total synthesis. Since the serological activity of native polyglutamic acid is a consequence of its structure, the structural features responsible for this activity can be established through the study of model compounds containing γ-glutamyl residues (*6*). It has also been suggested that the distance between free (α) carboxyl groups in native polyglutamic acid is important for serological activity (*7*). To test this hypothesis, a number of γ-glutamyl-containing polypeptides separated by α-, β-, or γ-amino acid residues are being studied (*6, 8*). A polypeptide where γ-glutamyl and β-aspartyl residues alternate has been synthesized and the secondary structure has been studied. This polypeptide has a higher molecular weight than previously synthesized sequential polypeptides using the pentachlorophenyl ester procedure (*8–11*).

To synthesize poly-β-(γ-L-glutamyl)-L-aspartic acid, it was necessary to prepare the active ester of the repeating dipeptide unit, α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid pentachlorophenyl ester hydrochloride (VI), as shown in the scheme.

The *N*-carbobenzoxy-α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid methyl ester (III) was prepared from *N*-carbobenzoxy-α-*t*-butyl-L-glutamic acid pentachlorophenyl ester (I) and α-*t*-butyl-L-aspartic acid methyl ester hydrochloride (II) in the usual manner. Compound II was obtained from *N*-carbobenzoxy-α-*t*-butyl-L-aspartic acid methyl ester by catalytic hydrogenolysis. Dipeptide derivative III was saponified to *N*-carbobenzoxy-α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid (IV) and converted to *N*-carbobenzoxy-α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid pentachlorophenyl ester (V) with pentachlorophenol and dicyclohexylcarbodiimide (*9*). Its infrared spectrum showed the characteristic bands for pentachlorophenyl ester at 5.6 μm and the doublet 7.2 and 7.4 μm, Catalytic hydrogenolysis of this dipeptide pentachlorophenyl derivative V in the presence of 1 equiv. of hydrogen chloride (*9*) resulted in the desired active dipeptide unit VI, in excellent yield. The didpeptide pentachlorophenyl ester V was also

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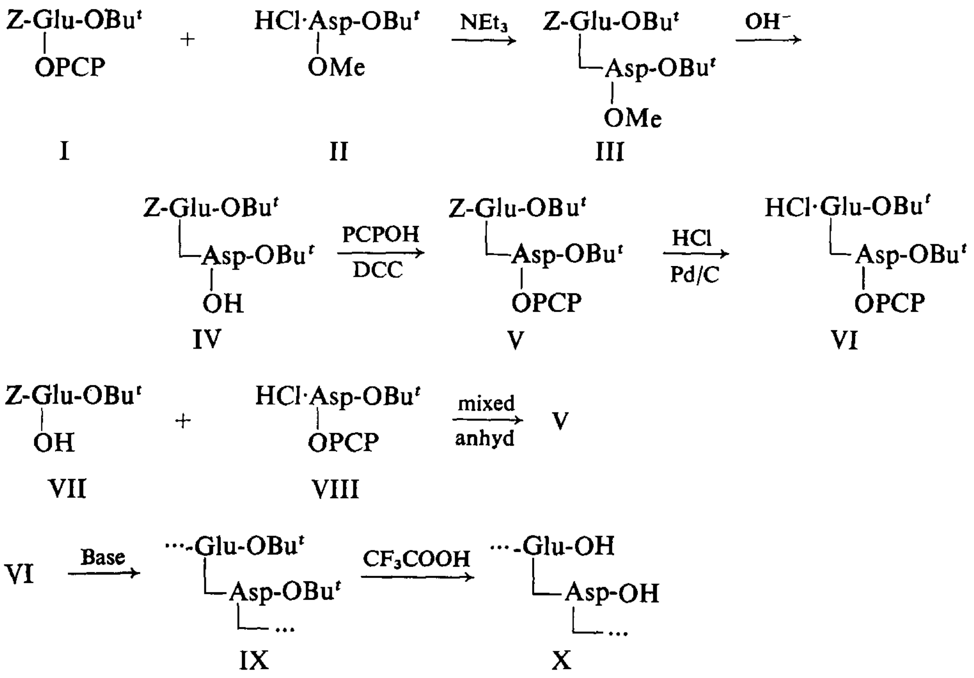
SCHEME



prepared from *N*-carbobenzoxy-α-*t*-butyl-L-glutamic acid (VII) and α-*t*-butyl-L-aspartic acid pentachlorophenyl ester hydrochloride salt (VIII) by the mixed anhydride procedure (*12*). Fifty percent solution of VI in purified dimethylformamide was polymerized in the presence of various amounts of purified triethylamine or *N*-methylmorpholine to get poly-α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid (IX) as shown in the scheme. The results of these polymerization experiments are tabulated in Table 1.

The polypeptide-*t*-butyl ester IX was treated with 90% aqueous trifluoroacetic acid to produce poly-β-(γ-L-glutamyl)-L-aspartic acid (X) in 80% yield. The absence of a peak at 11.8 μm in the infrared spectrum indicated the removal of the *t*-butyl ester group. In contrast to poly-α-aspartic acid and poly-α-glutamic acid this polypeptide is highly water-soluble. In this respect, it resembles native poly-γ-glutamic acid and

TABLE 1

POLYMERIZATION OF α-*t*-BUTYL-L-GLUTAMYL-α-*t*-BUTYL-L-ASPARTIC ACID PENTACHLOROPHENYL ESTER HYDROCHLORIDE IN DIMETHYLFORMAMIDE

Base	Time (days)	Yield of IX	Yield of X	$\bar{M}$W of X
TEA 2.5 equiv	1	43	80	28,000
TEA 3.1 equiv	3	50	80	21,000
NMM 2.2 equiv	3	64	80	7,000

poly-β-aspartic acid. The molecular weight of X was determined in the ultracentrifuge by the sedimentation equilibrium method (*13*) and these results are included in Table 1. Table 1 indicates that the highest molecular weight was obtained by using 2.5 equiv of triethylamine. The *N*-methylmorpholine produced a polymer with lower molecular weight but higher in yield.

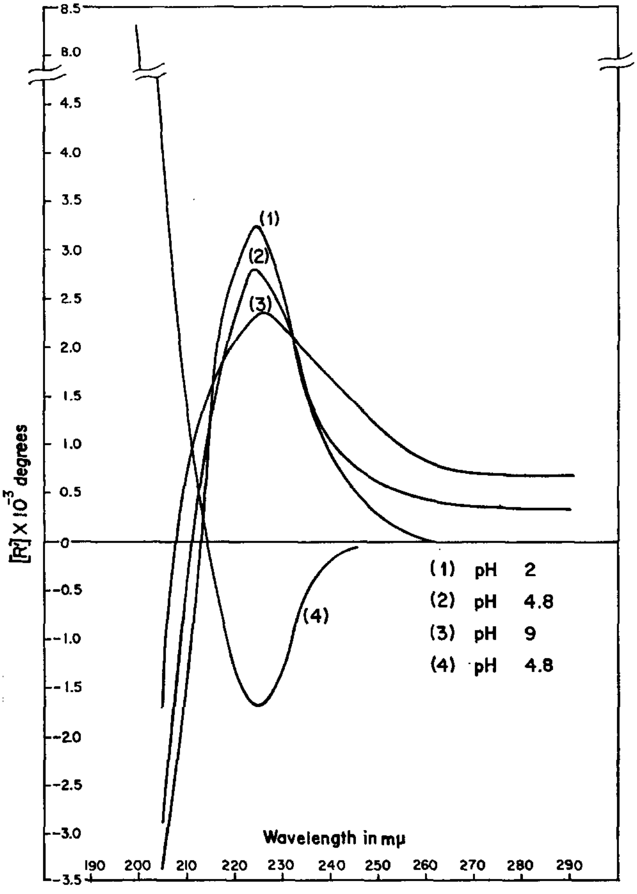


FIG. 1. ORD of poly-β-(γ-glutamyl)-aspartic acid, L (curves 1, 2 and 3) and D (curve 4) isomers in aqueous solution.

Poly-β-(γ-D-glutamyl)-D-aspartic acid was prepared similarly from dipeptide pentachlorophenyl ester V which was obtained from the D-isomer of VII and VIII through the mixed anhydride procedure.

Optical rotatory dispersion (ORD) and circular dichroism (CD) studies on poly-β(γ-L-glutamyl)-L-aspartic acid (X) in water at various pH values are shown in Figs. 1 and 2, respectively. The mean residue weight is one-half of the molecular weight of the repeating dipeptide unit. The possible origin of the 225-nm band in the ORD and the 203-nm band in the CD may be due in part to the n-π^* transition of the carboxyl chromophore in the side chain. Each peak persists throughout the entire pH range studied and gradually loses its intensity with increasing addition of base, but does not disappear. At pH 2, the carboxyl side chain is un-ionized whereas at pH 4.8 and higher it is essentially ionized. Therefore, the decrease of $[R']$ at 225 nm and the decrease of $[\theta]$ at 203 nm could be due to the neutralization of the carboxyl side chain. This same phenomenon has been observed for poly-β-L-aspartic acid (*14*) and poly-γ-L-glutamic acid (*15*). When the carboxyl group is one or two methylenes away from the peptide backbone as in poly-α-L-aspartic acid and poly-α-L-glutamic acid, the 203 nm peak does not seem to be present, and if it were there, this band would be masked by the peptide Cotton effect (*16*). In addition, assignment of this band to the carboxyl group would imply a high degree of optical activity due to the adjacent asymmetric carbon atom. It is suggested that under the condition of this study, poly-β-(γ-glutamyl)-aspartic acid has no special conformational order and that the 225-nm band in the ORD and the 203-nm band in CD may in part be due to the carboxyl chromophore in the side chain.

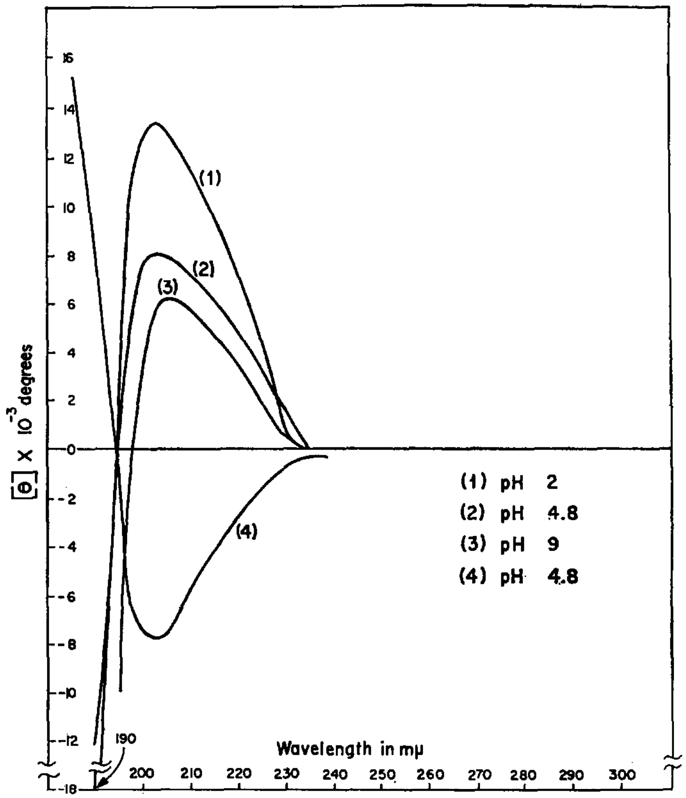


FIG. 2. CD of poly-β-(γ-glutamyl)-aspartic acid, L (curves 1, 2, and 3) and D (curve 4) isomers in aqueous solution.

The serological reaction of poly-β-(γ-glutamyl)-aspartic acid (D and L) with antiserum against *Bacillus anthracis* will be reported elsewhere.

EXPERIMENTAL

All melting points are uncorrected. The microanalyses were carried out either by Drs. G. Weiler and F. B. Strauss, Oxford, England, or by Schwarzkopf Microanalytical Laboratory, Woodside, NY. Infrared spectra were taken in potassium bromide pellets on a Perkin-Elmer Model 137 spectrophotometer.

N-*Carbobenzoxy-α-*t*-butyl-*L*-glutamyl-α-*t*-butyl-*L*-aspartic acid methyl ester* (*III*). A mixture of 3 g (5.12 mmoles) of *N*-carbobenzoxy-α-*t*-butyl-L-glutamic acid pentachlorophenyl ester (I), 0.486 g (5.12 mmoles) of 2-hydroxypyridine, 1.149 g (5.12 mmoles) of α-*t*-butyl-β-methyl-L-aspartic acid hydrochloride (II), 0.67 ml (5.12 mmoles) of triethylamine and 50 ml of methylene chloride was stirred at room temperature for 24 hr. The reaction mixture was filtered and the filtrate was successively washed three times with 20-ml portions of cold dilute sodium hydroxide, three times with 30-ml portions of *N*-hydrochloric acid, 30-ml portions of 5% sodium bicarbonate, twice with 30-ml portions of water, dried over sodium sulfate, and evaporated under reduced pressure; yield 1.74 g (65%), mp 83-85°C. The crude product was chromatographed on a silica gel column and recrystallized four times from ether–petroleum ether; yield 1.0 g (37.3%), mp 87–88°C. The ir spectrum showed peaks at 5.79–5.83 (methyl and *t*-butyl ester), a shoulder at 5.88 (carbobenzoxy), 6.02 (amide I), 6.54 (amide II) and 11.80 μm (*t*-butyl).

Anal. Calcd for $C_{26}H_{38}N_2O_9$ C, 59.75; H, 7.33; N, 5.36. Found: C, 59.85; H, 7.29; N, 5.40.

N-*Carbobenzoxy-α-*t*-butyl-*L*-glutamyl-α-*t*-butyl-*L*-aspartic acid* (*IV*). A solution of 1.95 g (3.73 mmoles) of *N*-carbobenzoxy-α-*t*-butyl-L-glutamyl-α-*t*-butyl-β-methyl-L-aspartic acid (III) and 8.2 ml of 0.5 *N* sodium hydroxide in 8.2 ml of acetone was stirred at room temperature for 1 hr, and then concentrated under reduced pressure. The residue was extracted with ethyl acetate, and ethyl acetate solution was back-extracted four times with 3-ml portions of 0.5 *N* sodium hydroxide. The combined alkaline extracts were cooled, acidified to pH 1 with 0.5 *N* hydrochloric acid, and extracted twice with 5-ml portions of ethyl acetate. The ethyl acetate layers were washed twice with 4-ml portions of water, dried over sodium sulfate, and evaporated under reduced pressure; yield 1.8 g (95%) as an oil.

N-*Carbobenzoxy-α-*t*-butyl-*L*-glutamyl-α-*t*-butyl-*L*-aspartic acid pentachlorophenyl ester* (*V*). *Method I.* A mixture of 1.8 g (3.55 mmoles) of *N*-carbobenzoxy-α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid (IV), 0.8 g (3.9 mmoles) of dicyclohexylcarbodiimide and 0.95 g (3.55 mmoles) of pentachlorophenol in 90 ml of methylene chloride was stirred at 0°C for 3 hr and overnight at room temperature. Five drops of acetic acid were added and stirring was continued for 30 min. The precipitated dicyclohexylurea (DCU) was filtered and the filtrate was concentrated under reduced pressure, The solid was dissolved in a minimum volume of hot ethyl acetate, cooled to room temperature, and filtered to remove residual DCU. The filtrate was concentrated in vacuum; yield 1.9 g (71%), mp 133–135°C. The crude product was recrystallized from methanol; yield 1.4 g (52%), mp 142.5–143.5°C, $[\alpha]_D^{21}$ 39.8° (c = 2, chloroform). The ir spectrum showed peaks at 5.60 (pentachlorophenyl ester), a shoulder at 5.88 (carbobenzoxy), 6.08 (amide I), 6.50 (amide II) and 11.85 μm (*t*-butyl).

Anal. Calcd for $C_{31}H_{35}N_2O_9Cl_5$: C, 49.18; H, 4.66; N, 3.69. Found: C, 49.11; H, 4.78; N, 3.52.

Method II. To 6.01 g (17.8 mmoles) of *N*-carbobenzoxy-α-*t*-butyl-L-glutamic acid (VII) in 130 ml of athyl acetate, cooled to –20°C in a methanol–dry ice bath, 1.94 ml (17.8 mmoles) of *N*-methylmorpholine and 2.48 ml (19.6 mmoles) of isobutyl chloroformate were added consecutively. The mixture was stirred at –20°C for 15 min and 4.64 g (17.8 mmoles) of α-*t*-butyl-L-aspartic acid pentachlorophenyl ester hydrochloride (VIII) was added followed by 2.5 ml (17.8 mmoles) of triethylamine. After stirring for 3 hr at –20°C, the reaction mixture was concentrated under reduced pressure and the residue was distributed between 250 ml of chloroform and 125 ml of distilled water. The chloroform layer was washed with 80 ml of 0.25 *N* sodium bicarbonate, 80 ml of distilled water, 80 ml of 0.5 *N* hydrochloric acid and three times with 80 ml of distilled water, dried over sodium sulfate and concentrated under vacuum; yield 12.0 g (89.0%), mp 131–132°C. The crude product was recrystallized from 80 ml of hot methanol; yield 10.8 g (80%), mp 141.5–142.5°C, $[\alpha]_D^{23}$ 39.4° (c = 2, chloroform). Its ir spectrum was superimposable with that of the compound prepared by Method I.

N-*Carbobenzoxy-α-*t*-butyl-*D*-glutamyl-α-*t*-butyl-*D*-aspartic acid pentachlorophenyl ester.* It was prepared similarly as in Method II in 71.9% yield, mp 141.5–142.5°C, $[\alpha]_D^{23}$ –39.1° (c = 1.81, chloroform). Its ir spectrum was superimposable on that of the L-isomer.

Anal. Calcd for $C_{31}H_{35}N_2O_9Cl_5$: C, 49.18; H, 4.66; N, 3.69; Cl, 23.41. Found: C, 49.05; H, 4.53; N, 3.74; Cl, 23.45.

*α-*t*-Butyl-*L*-glutamyl-α-*t*-butyl-*L*-aspartic acid pentachlorophenyl ester hydrochloride (VI).* To a prehydrogenated suspension of 150 mg of 10% palladium on charcoal in 1 ml of glacial acetic acid and 50 ml of methanol, 1.514 g (2 mmoles) of *N*-carbobenzoxy-α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid pentachlorophenyl ester (V) and 0.57 ml of methanol containing 76.2 mg (2.09 mmoles) of hydrogen chloride were added. Hydrogenation was continued for 10 min, during which time one-half of the theoretical amount of hydrogen (22.4 ml) was absorbed. The reaction mixture was filtered, the filtrate concentrated to 2 ml under reduced pressure, and diluted with anhydrous ether to the turbidity point. On standing overnight at –10°C, the product crystallized; it was filtered, washed with anhydrous ether, and dried in vacuum; yield 1.14 g (86.8%), mp 145.5–146.5°C, $[\alpha]_D^{23}$ 13.7° (c = 2, methanol). The ir spectrum showed absence of carbobenzoxy peak at 5.88 μm.

Anal. Calcd for $C_{23}H_{30}N_2O_7Cl_6$: C, 41.90; H, 4.59; N, 4.25; Cl, 32.27. Found: C, 41.63 H, 4.87; N, 4.35; Cl, 32.20.

*α-*t*-Butyl-*D*-glutamyl-α-*t*-butyl-*D*-aspartic acid pentachlorophenyl ester hydrochloride.* It was prepared similarly in 83% yield, mp 146–147°C, $[\alpha]_D^{23}$ –13.6° (c = 1.49, methanol). Its ir spectrum was superimposable on that of the L-isomer. Found: C, 41.16; H, 4.40; N, 4.28; Cl, 32.29.

*Poly-α-*t*-buty-*L*-glutamyl-α-*t*-butyl-*L*-aspartic acid (IX).* A mixture of 0.827 g (1.26 mmoles) of α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid pentachlorophenyl ester hydrochloride (VI), 1.2 ml of purified DMF and 0.44 ml of triethylamine was shaken for 24 hr at room temperature. The reaction mixture was diluted with 300 ml of ether, filtered, and washed, first with water, to remove triethylamine hydrochloride, then with methanol to remove low molecular-weight polymer (28%), and finally with ether. The white product was dried for 16 hr at 60°C (0.1 mm). The yield was 0.19 g (42.9 %), the ir spectrum showed peaks at 6.05 (amide I), 6.55 (amide II), and 11.85 μm (*t*-butyl). The pentachlorophenyl ester peak at 5.62 μm was absent.

Anal. Calcd for $(C_{17}H_{28}N_2O_6)_n$: C, 57.29; H, 7.91; N, 7.86. Found: C, 57.18; H, 7.87; N, 7.71.

Polymerization of 1.00 g of hydrochloride salt VI in 1.6 ml of DMF with 0.37 ml of *N*-methylmorpholine (2.2 equiv) was carried out at room temperature for 3 days. The reaction mixture was worked up as described above; yield 0.35 g (63.3 %). Its ir spectrum was superimposable on that of the above preparation. The intrinsic viscosity was 0.143 dl/g in dichloroacitic acid. Found: C, 56.69; H, 7.52; N, 7.88.

*Poly-α-*t*-butyl-*D*-glutamyl-α-*t*-butyl-*D*-aspartic acid.* It was prepared similarly in 51 % yield. Its ir spectrum was superimposable on that of the L-isomer. Found: C, 56.64; H, 7.99; N, 7.80.

*Poly-β-(γ-*L*-glutamyl)-*L*-aspartic acid* (X). A solution of 0.169 g (0.475 mmoles) of poly-α-*t*-butyl-L-glutamyl-α-*t*-butyl-L-aspartic acid (IX) in 2.2 ml of 90 % trifluoroacetic acid was allowed to stand at room temperature for 50 min, then diluted with 140 ml of ether and centrifuged. The polymer was washed three times with ether and dried under vacuum at 60°C; yield 92.7 mg (80 %). The product (70 mg) was dissolved in 13 ml of water and dialyzed against three 200-ml portions of water for 30 hr. The dialyzed solution. was filtered and lyophilized; yield 15 mg (22.4 %). There was no peak at 11.85 μm in the ir spectrum, which indicates the removal of the *t*-butyl group. The $\bar{M}W$ was 28,000 as determined by sedimentation equilibrium.

Anal. Calcd for $(C_9H_{12}N_2O_6 \cdot H_2O)_n$; C, 41.32; H, 5.96; N, 10.78. Found: C, 41.22; H, 5.38; N, 10.68.

*Poly-β-(γ-*D*-glutamyl)-*D*-aspartic Acid.* It was prepared similarly in 80 % yield. Its ir spectrum was superimposable on that of the L-isomer. The product (100 mg) was dissolved in 19 ml of water, dialyzed and lyophilized; yield 61 mg (61 %), $\bar{M}W$ 9,500. Found: C, 41.00; H, 5.60; N, 10.70.

Weight-average molecular weights. The weight-average molecular weights ($\bar{M}W$) were determined in the Spinco Model E analytical ultracentrifuge by the sedimentation equilibrium method in 0.1 *M* aqueous KCL. The measurements were made at a rotor speed of 12,580–21,400 at 27°C and in a concentration range of 0.2–0.5 %. The $\bar{M}W$ values are summarized in Table 1.

ORD and DC studies. Optical rotatory dispersion and circular dichroism measurements were made with the Cary 60 automatic recording spectropolarimeter. The cell paths used were between 0.1–0.5 mm and concentrations were between 0.10 and 0.18 g of polymer per 100 ml of buffer, obtained from Fisher Scientific Co., at the desired pH values.

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