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# Helix–Coil Stability Constants for the Naturally Occurring Amino Acids in Water. 16. Aspartic Acid Parameters from Random Poly(hydroxybutylglutamine-*co*-L-aspartic acid)[1]

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ABSTRACT: The synthesis and characterization of water-soluble random copolymers containing L-aspartic acid with $N^5$-(4-hydroxybutyl)-L-glutamine, and the thermally induced helix–coil transitions of these copolymers in water and in 0.1 N KCl, are described. The incorporation of L-aspartic acid was found to decrease the helix content of the polymer at both high and low pH, in water and also in 0.1 N KCl. The Zimm–Bragg parameters $\sigma$ and $s$ for the helix–coil transition in poly(L-aspartic acid) in water and in 0.1 N KCl were deduced from an analysis of the melting curves of the copolymers in the manner described in earlier papers. Corrections were made for the presence of a small amount of racemized aspartic acid, using data from random copolymers containing D-aspartic acid as the guest residue. The computed values of $s$ indicate that L-aspartic acid destabilizes helical sequences at all temperatures in the range of 0–70 °C. Titrations of the copolymers and of *N*-acetyl-*N'*-methyl-L-aspartic acid amide in 0.1 N KCl are described.

There have been several investigations of the conformation of poly(L-aspartic acid) in aqueous solutions.[3-9] Little information, however, is available for the conformational preference of the L-aspartic acid residue, because of difficulties in the synthesis and quantitative analysis of polymers containing L-aspartic acid. These investigations suggest that neutral poly(L-aspartic acid) forms a right-handed $\alpha$ helix in water with a less organized helical structure than its homologue poly(L-glutamic acid)[6,7] and does not display a detectable cooperative helix–coil transition throughout its range of ionization.[8]

The unusually large tendency for aspartic acid to undergo racemization and transamidation introduces difficulties in the synthesis of poly(L-aspartic acid).[3-5] Difficulties in the analysis of the properties of this homopolymer arise from the presence of charged residues which complicate the interpretation of the results.[7,8] To eliminate these difficulties and to gain insight into the conformational behavior of L-aspartic acid residues in proteins, water-soluble random copolymers of L-aspartic acid and a nonionizable "host", $N^5$-(4-hydroxybutyl)-L-glutamine (HBG), have been synthesized. By application of the host–guest technique described and applied in previous papers of this series, the most recent one being that of Konishi et al.,[10] the Zimm–Bragg helix–coil stability constants,[11] $\sigma$ and $s$, of L-aspartic acid were determined in both the charged and uncharged states. Random copolymers of $N^5$-(4-hydroxybutyl)-L-glutamine with D-aspartic acid were also synthesized and their helix–coil stability constants were determined in order to correct for the contribution from the racemized residues to the helix–coil stability constants of L-aspartic acid.

The results presented here indicate that L-aspartic acid is a pronounced helix breaker in water over the temperature range 0–70 °C and confirm the earlier qualitative work that poly(L-aspartic acid) has a low helix content in aqueous solution. They also demonstrate that there is a difference between the helix-forming abilities of charged and uncharged residues of aspartic acid, similar to the cases of L-glutamic acid and L-lysine in papers IX and XI of this series;[12,13] i.e., charged L-aspartic acid is intrinsically a stronger helix breaker than is the uncharged form.

The synthesis of water-soluble random copolymers of L- and D-aspartic acids, respectively, with $N^5$-(4-hydroxybutyl)-L-glutamine, and their fractionation, are described in section I. The characterization of these copolymers and their melting and titration behavior are presented in section II. Finally, the data are analyzed to determine the helix–coil stability parameters of L-aspartic acid in their uncharged and charged forms in water and in 0.1 N KCl in section III. These results are then compared with previous results for polypeptides and proteins.

## I. Experimental Section. Preparation and Characterization of the Copolymers

The copolymers were prepared from the *N*-carboxyanhydrides of $\gamma$-benzyl L-glutamate and $\beta$-*tert*-butyl L-aspartate (or D-aspartate)

in dioxane with sodium methoxide as initiator. The *tert*-butyl blocking groups were then removed by treatment with trifluoroacetic acid and, lastly, the γ-benzyl ester groups were substituted by 4-hydroxybutylamide groups to yield copolymers of $N^5$-(4-hydroxybutyl)-L-glutamine with L-aspartic acid (or D-aspartic acid).

**(A) Materials.** The α-benzyloxycarbonyl β-*tert*-butyl L-aspartate was obtained from Bachem, Marina Del Rey, Calif. The D-aspartic acid and the L-glutamic acid were purchased from Aldrich Chemical Co., Inc. L-Aspartic acid, L-glutamic acid, and D,L-aspartic acid, used as a standard to test for optical purity, were products of Schwartz/Mann. All other reagents and solvents were identical in quality and preparation to those used in papers IX[12] and XIV[14] of this series.

Poly[$N^5$-(4-hydroxybutyl)-L-glutamine], poly(HBG), of degree of polymerization $\overline{DP}_w$ = 720 and 200 were fractions V and VIB, respectively, of paper II[15] of this series.

**(B) Synthesis. *N*-Carboxyanhydrides.** α-Benzyloxycarbonyl β-*tert*-butyl L-aspartate was hydrogenated over Pd (10%) on charcoal in anhydrous methanol.[16] After precipitation with ethyl acetate, pure β-*tert*-butyl L-aspartate was obtained in 86% yield: mp 195–196 °C dec; $[\alpha]^{20}_D$ +9.1 (*c*, 1.0, $CH_3COOH$ 90%) [lit.[17] mp 189–190 °C; $[\alpha]_D$ +8.5 (*c*, 1.0, $CH_3COOH$ 90%)] [lit.[18] mp 198–199 °C, $[\alpha]_D$ +8.5 (*c*, 1.3, $H_2O$)]. β-*tert*-Butyl aspartate was reacted with phosgene in tetrahydrofuran, following the general procedure described by Hirschmann et al.[19] Two crystallizations of this product from ethyl acetate–hexane gave a 55% yield of a snow-white solid of the *N*-carboxyanhydride of β-*tert*-butyl L-aspartate: mp 138–139 °C dec; $[\alpha]_D$ −35.4 (*c*, 0.98, ethyl acetate) [lit.[18] mp 138–140 °C $[\alpha]_D$ −35.6 (*c*, 2.0, EtOAc)].

The stereochemical purity of the *N*-carboxyanhydride of β-*tert*-butyl L-aspartate was checked by the Manning–Moore dipeptide procedure,[20] and it was found to contain less than 0.1 mol % of the D isomer.

β-*tert*-Butyl D-aspartate [mp 193–194 °C, $[\alpha]^{20}_D$ −8.8 (*c*, 1.2, $H_2O$)] and its *N*-carboxyanhydride [mp 138–139 °C, $[\alpha]_D$ +38.0 (*c*, 2.0, EtOAc)] were prepared in the same way as the corresponding compounds of L-aspartate. The *N*-carboxyanhydride of β-*tert*-butyl D-aspartate was also found to contain less than 0.1 mol % of the L isomer.

γ-Benzyl L-glutamate *N*-carboxyanhydride was prepared, as in paper VII[21] of this series, according to the general method described by Hirschmann et al.[19]

**Poly[γ-benzyl L-glutamate-*co*-β-*tert*-butyl L-aspartate], Poly[Glu(OBzl),L-Asp(OBu$^t$)], Copolymers A-L-I and A-L-II.** Random copolymers of γ-benzyl L-glutamate with β-*tert*-butyl L-aspartate were synthesized by polymerization of the *N*-carboxyanhydrides in dioxane with sodium methoxide as initiator.[12] The ratios of the aspartate to glutamate *N*-carboxyanhydrides were 1:9 for copolymer A-L-I and 3:17 for copolymer A-L-II, respectively. The reactions, monitored as described in paper X[22] of this series, were more than 90% complete within a few hours. After completion of the polymerization, the viscous solution was poured slowly into absolute ethanol with stirring. The solid (in 89% yield) was collected on a suction filter, washed with ethanol, and dried in vacuo (over $P_2O_5$/KOH). The chain lengths of these polymers (determined, roughly, by the viscosity–molecular weight relationship of Fujita et al.[23]) are given in Table I.

**Poly[γ-benzyl L-glutamate-*co*-L-aspartic acid], Poly[Glu(OBzl),L-Asp], Copolymers B-L-I and B-L-II.** In order to remove the *tert*-butyl protecting groups from the aspartic acid residues, poly[Glu(OBzl),L-Asp(OBu$^t$)] was dissolved in trifluoroacetic acid and allowed to stand at room temperature for 2 h. Then the solution was poured into ether. The fibrous white precipitate was filtered, washed with ether, and dried over $P_2O_5$ in vacuo. The yield was 96%.

From the fact that, in the *final* copolymers, the Asp content (determined by amino acid analysis) agreed with the COOH content (determined by titration), we conclude that the *tert*-butyl groups were removed completely (within the experimental errors in these two types of analyses).

**Poly[$N^5$-(4-hydroxybutyl)-L-glutamine-*co*-L-aspartic acid], Poly[HBG,L-Asp), Copolymers L-I and L-II.** In order to convert poly[Glu(OBzl),L-Asp] to the corresponding water-soluble copolymers, the copolymer was treated with 4-amino-1-butanol as described in paper IV[24] of this series. The copolymer was swollen in dioxane at room temperature for several hours, and 1-hydroxybenzotriazole (1.2 mol/mol of amino acid residues in the polymer) was added to the solution to catalyze[25] the aminolysis of the benzyl ester groups, thereby shortening the time of exposure of the polymer to base and expecting to reduce the extent of both racemization of the aspartate residues and chain degradation. 4-Amino-1-butanol was then added dropwise to the reaction mixture at 50 °C, and the course of the aminolysis was monitored by assaying for unexchanged γ-benzyl ester groups, as described in paper X[22] of this series. The reaction was terminated when more than 99.5% of the benzyl ester groups had been exchanged. The reaction mixture was then poured into a rapidly stirred, ice-cold, dilute, aqueous solution of hydrochloric acid, the acid being in sufficient excess to provide a concentration of about 0.1 N in the reaction mixture. The solution was then dialyzed extensively against 0.01 N aqueous hydrochloric acid until amines could no longer be detected by a ninhydrin test on the dialyzate[26] and then against water until neutral. After lyophilization, a yield of about 85%, based on the number of moles of poly[Glu(OBzl),Asp], was obtained. All polymers remained colorless throughout the entire synthesis.

**Poly[γ-benzyl L-glutamate-*co*-β-*tert*-butyl D-aspartate], Poly[Glu(OBzl),D-Asp(OBu$^t$)], Copolymer A-D-I.** A random copolymer of γ-benzyl L-glutamate with β-*tert*-butyl D-aspartate was synthesized in the same manner as for poly[Glu(OBzl),L-Asp(OBu$^t$)]. The ratio of the aspartate to glutamate *N*-carboxyanhydride was 1:9.

**Poly[γ-benzyl L-glutamate-*co*-D-aspartic acid], Poly[Glu(OBzl),D-Asp], Copolymer B-D-1, and Poly[$N^5$-(4-hydroxybutyl)-L-glutamine-*co*-D-aspartic acid], Poly[HBG,D-Asp], Copolymer D-I.** Poly[Glu(OBzl),D-Asp] was prepared, using the same procedure as for poly[Glu(OBzl),L-Asp]. A random copolymer of $N^5$-(4-hydroxybutyl)-L-glutamine with D-aspartic acid was obtained using poly[Glu(OBzl),D-Asp] in the same manner as used for poly[HBG,L-Asp].

**(C) Fractionation.** The water-soluble copolymers (L-I, L-II, and D-I) were fractionated with methanol and ether by the procedure described in paper II[15] in this series. After fractionation, the polymers were dissolved in water, lyophilized, and dried in vacuo over $P_2O_5$. Only those fractions which were used for the analysis of σ and *s* are listed in Table II.

**Table I**
**Compositions and Chain Lengths of the Unfractionated Copolymers**

| Polymer No. | *t*-Bu-Asp content of reaction mixture, mol % | Av mol wt[a] × $10^{-3}$ | $\overline{DP}$ |
|---|---|---|---|
| A-L-I | 10 (L-Asp) | 270 | 1260 |
| A-L-II | 15 (L-Asp) | 165 | 780 |
| A-D-I | 10 (D-Asp) | 137 | 640 |

[a] By viscometry, using the relation of Fujita et al.[23] for polymers in dichloroacetic acid.

**Table II**
**Characterization of the Fractionated Copolymers**

| Fraction[a] | Aspartic acid content,[b] mol % | Racemization of L-aspartic acid residues,[c] mol % | $\overline{M}_w$[d] × $10^{-3}$ | $\overline{M}_z/\overline{M}_w$ | $\overline{DP}_w$ |
|---|---|---|---|---|---|
| L-I-2 | 8.1 | 14.4 | 133 | 1.01 | 690 |
| L-I-5 | 8.3 | 14.5 | 99 | 1.00 | 515 |
| L-I-7 | 9.1 | 15.7 | 37 | 1.11 | 190 |
| L-II-3 | 13.1 | 15.1 | 207 | 1.14 | 1100 |
| L-II-5 | 14.2 | 13.1 | 108 | 1.07 | 575 |
| L-II-6 | 13.6 | 14.8 | 70 | 1.00 | 370 |
| Fraction[a] | Aspartic acid content,[b] mol % | Racemization of D-aspartic acid residues,[e] mol % | $\overline{M}_w$[b] × $10^{-3}$ | $\overline{M}_z/\overline{M}_w$[c] | $\overline{DP}_w$ |
| D-I-2 | 8.2 | 12.8 | 172 | 1.04 | 890 |
| D-I-3 | 8.1 | 15.0 | 133 | 0.99 | 710 |
| D-I-4 | 10.6 | 17.4 | 81 | 1.00 | 430 |
| D-I-5 | 16.9 | 14.3 | 29 | 1.15 | 155 |

[a] The numerals 2, 3, 4, etc., designate fractions obtained from L-I, L-II, and D-I, respectively. [b] This is the total Asp content (i.e., D + L). [c] This is the percentage of total Asp that is present in the D form. [d] Obtained by sedimentation equilibrium (with extrapolation to zero concentration). [e] This is the percentage of total Asp that is present in the L form.

**(D) Analytical Methods. Amino Acid Analysis.** The amino acid composition of all copolymer fractions was determined on a Technicon TMS amino acid analyzer. Each copolymer fraction was hydrolyzed, according to the procedure of Moore and Stein,[27] in 6 N HCl at 110 °C for 24 h in degassed sealed ampules. It is possible for artifacts to form during the removal of hydrochloric acid from the hydrolyzates.[28,29] These artifacts would be γ and β esters of glutamic acid and aspartic acid, respectively, with 4-amino-1-butanol.[28,30] In order to avoid formation of such artifacts, the usual evaporation of the hydrochloric acid was omitted; instead, the hydrolyzates were diluted four to six times with water. Thus, the HCl present in the aliquots that were applied to the analyzer did not affect[31] the separation or the analysis of the amino acids with a citrate eluting buffer. The average experimental error in the determination of the amino acid composition is estimated to be ±4%.

**Viscometry.** Viscosity measurements on copolymers A-L-I, A-L-II, and A-D-I in dichloroacetic acid were carried out with a Cannon-Ubbelohde semimicro dilution viscometer at 25.0 ± 0.1 °C. Using the viscosity–molecular weight relationship of Fujita et al.,[23] the molecular weights of these copolymers were determined roughly.

**Determination of Molecular Weights.** Weight-average and z-average molecular weights of the water-soluble fractions were determined by the conventional sedimentation-equilibrium method using a Beckman Model E analytical ultracentrifuge.[32] The polymers were dissolved in dilute aqueous hydrochloric acid (pH 1.5) in order to suppress the ionization of the β-carboxyl groups of the aspartic residues and, thus, eliminate polyelectrolyte effects. The weight-average molecular weights were obtained by extrapolation to zero concentration of the apparent molecular weights determined at three concentrations, viz., ca. 0.1, 0.2, and 0.3%. The z-average molecular weights were calculated at each concentration by the method described earlier,[24] and the reported values of $\overline{M}_z/\overline{M}_w$ were calculated from values obtained at the lowest solute concentrations studied. The partial specific volumes ($\bar{v}$) of the copolymers were calculated from their amino acid contents with the method described by Cohn and Edsall,[33] using the value of $\bar{v}$ = 0.816 cm³/g from paper II[15] for HBG residues. The average experimental error in the determination of the molecular weight is estimated to be ±5%.

**Assay for Extent of Racemization.** The stereoisomeric purity of the amino acids in the starting materials and in the copolymers poly[HBG,D- or L-Asp(OBu$^t$)] and poly[HBG,D- or L-Asp] was checked by the L-leucyl-dipeptide method of Manning and Moore.[20] It should be noted that poly[HBG,D- or L-Asp(OBu$^t$)] was *not* used for the physicochemical studies described below. The aspartic and glutamic acids were isolated from the acid hydrolyzates of the copolymers prior to derivatization, as described in paper X.[22] The amino acids were separated by ion-exchange chromatography on a 60 mm × 5 mm column of Dowex AG 1-X8 (200–400 mesh) in the acetate form by elution with 0.5 N acetic acid at a flow rate of 14 mL/h.

**Assay for β-Carboxamide Linkages.** The assay for aspartyl-β-carboxamide linkages in the copolymers was carried out by the method of Miller and Loudon[34] for sequencing proteins from the C terminus. The procedure involves three steps. The first is the formation of an *O*-pivaloylhydroxamate with all α- and β-carboxyl groups in the copolymer, the second is a Lossen rearrangement at pH 8.5, and the third is an acid hydrolysis. These treatments convert the amino acid residues carrying free α carboxyls in the copolymer (aspartic acid in β-carboxamide linkage and C-terminal residues) to aldehydes, which are determined indirectly by the loss of the corresponding amino acids in amino acid analysis. Residues carrying free β carboxyls in the copolymer (aspartic acid in α-carboxamide linkage) are recovered unchanged.[35] Miller and Loudon[34,35] applied their method to our copolymers and reported the details of the method and the results.

**Titrations.** The copolymers were titrated to determine the number of free carboxyl groups and their titration curves. The model compound *N*-acetyl-*N'*-methylaspartic acid amide was also titrated. The instrument used for the potentiometric titrations was an improved version of one that was reported previously[36] and is described in the Appendix. It is of high stability with negligible drift. The combined glass and reference electrodes (No. 6030-02) were the product of the Ingold Electrode Co. These were calibrated just before and after each use with phthalate and phosphate buffers made up according to Bates.[37]

Ten milliliters of solutions containing about 15 mg of sample dissolved in 0.1 N KCl were titrated. The apparent p$K$ at any degree of ionization was calculated using the equation[38]

$$pK_{app} = pH - \log[\alpha/(1 - \alpha)] \quad (1)$$

where α is the fraction ionized and is determined in the usual manner.[39] The other experimental conditions, and the procedure for correcting for the error in the determination of the end points, are the same as those used in paper IX of this series.[12]

**Optical Rotatory Dispersion and Circular Dichroism Measurement.** The optical rotatory dispersion (ORD) and circular dichroism (CD) measurements were made on a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment and on a Jasco J-20 automatic recording spectropolarimeter. Temperature control was maintained to within ±0.2 °C with water-jacketed quartz cells. The procedures were the same as those used previously.[24]

**Determination of Concentration.** The concentrations of all copolymer solutions were determined by micro-Kjeldahl analysis for nitrogen as described previously.[24] Multiple aliquots of each solution were analyzed. The error in this measurement was found to be ±3%.

## II. Results

**(A) Synthesis and Characterization of the Copolymers.** Peptides which contain aspartic acid residues are known to be racemized easily under basic conditions. One of the explanations for this behavior is that the electrophilic β-carboxyl group promotes the abstraction of the proton from the asymmetric α-carbon atom which undergoes base-catalyzed racemization.[40] Peptides which contain aspartic acid β esters, on the other hand, are known to undergo facile base-catalyzed transamidation reactions through intermediate succinimide derivatives.[41,42] During the aminolysis of the γ-benzyl glutamate, the copolymer is exposed to the basic amino butanol for about 4 days.

In order to check for racemization and transamidation, several syntheses were tried in preliminary experiments. In the first method, fully protected poly[Glu(OBzl),L-Asp(OBu$^t$)] was treated with 4-aminobutanol. After completion of the aminolysis, the *tert*-butyl ester groups were removed by exposure to aqueous HCl and the copolymer was isolated by the procedure used for poly[HBG,L-Glu(OBu$^t$)].[12] This copolymer was shown to have the same composition by amino acid analysis as the fully protected starting copolymer and also to contain less than 2% of the aspartic residues as D isomers. However, it had only about half of the expected titratable carboxyl groups. The number of carboxyl groups increased to about the expected value after 2 h in solution at pH 11 at room temperature, making the titration curve nonreversible. This behavior is reminiscent of the titration of succinimide derivates, e.g., ε-(aminosuccinyl)lysine.[43,44] The number of titratable carboxyl groups depended somewhat on the conditions used in the acid treatment after aminolysis, but neither 4 N aqueous HCl nor 90% trifluoroacetic acid would yield a copolymer with a titration behavior consistent with its aspartic acid content. These observations are at variance with a similar synthesis of a copolymer with glutamic acid,[12] which titrated as expected. Miller and Loudon[35] investigated this copolymer for the presence of α-carboxyl groups which would indicate aspartyl residues in β-carboxamide linkage and showed that 21% of the carboxyl groups originally titrated were in fact aspartic acid α-carboxyl groups. After exposure of the copolymer to base (pH 11) as mentioned above, 29% of the increased number of carboxyl groups were shown to be aspartic acid α-carboxyl groups. The nature of the base-sensitive groups which masked the aspartic acid carboxyl groups to titration was not determined, but these might be cyclic imides of β carboxyls with backbone amide groups. Such succinimide derivatives have been observed before with esterified aspartyl residues in peptides exposed to base[41,42] and are likely to be the intermediates in transamidation reactions between α- and β-peptide linkages.

In a second method, partially deprotected poly[Glu(OBzl),Asp] was treated with 4-aminobutanol. The resulting polymer had the correct number of titratable carboxyl groups as expected from its aspartic acid content. Exposure to aqueous base did not change the number of titratable

groups, and the titration curves were fully reversible. Miller and Loudon[35] showed that there were no aspartyl $\alpha$-carboxyls in this polymer, indicating that all of the aspartic acid residues were in $\alpha$-peptide linkages. However, about 15% of the aspartic acid residues of this copolymer were in the form of the D isomer while less than 2% of the glutamic acid residues were racemized, indicating a selectively increased racemization of the aspartic acid residues during this synthesis.

The second procedure was adopted for the preparation of the copolymers for the physicochemical studies because it gave no $\beta$-carboxamide linkages or masked carboxyl groups and because the presence of about 15% of the aspartic acid residues in the D form could be corrected for (in determining $\sigma$ and $s$ for L-Asp) by using melting data for poly[HBG,D-Asp] as discussed below.

Table I summarizes the composition and the average degree of polymerization ($\overline{DP}$) of the unfractionated, fully protected copolymers A-L-I, A-L-II, and A-D-I, and Table II shows the data for the fractions of the corresponding deprotected copolymers, L-I, L-II, and D-I, which were investigated to determine the helix–coil stability parameters. The usual decrease in $\overline{DP}$ occurred during the aminolysis.[22] The extent of chain degradation could be reduced by the addition of 1-hydroxybenzotriazole to the reaction mixture. When the aminolysis was carried out without 1-hydroxybenzotriazole, the poly(HBG,L-Asp) had only 60% of the original $\overline{DP}$ of poly[Glu(OBzl),L-Asp]. However, aminolysis in the presence of 1-hydroxybenzotriazole gave 80% of the original $\overline{DP}$.

The aspartic acid contents of the fractions of copolymers L-I and L-II are almost independent of chain length for a given (protected) copolymer parent. This indicates that there is little departure from randomness in these copolymers. While this criterion for near randomness has been used in previous papers of this series,[14] it was verified (in the case of copolymers containing methionine as the guest residue[14]) by determining the distribution of fragments obtained from a degradation of the copolymer with cyanogen bromide.[45] In addition, it has been demonstrated theoretically in paper I of this series[46] that small deviations from randomness do not influence the melting behavior of these copolymers.

On the other hand, the composition does vary significantly with chain length for fractions derived from copolymer D-I, indicating a larger departure from randomness in this case. This behavior probably arose because the reaction rates of the *N*-carboxyanhydrides of L and D amino acids are different.[47-49] However, the data for copolymer D-I were used only to compensate for the minor effects resulting from racemization in copolymers L-I and L-II (see below). For this reason, the possibility of a departure from randomness in fractions from D-I could be neglected.

The fact that the degree of racemization of each fraction derived from L-I, L-II, and D-I was almost the same indicates that racemization occurred randomly on the aspartic acid residues. In the Manning–Moore assay for optical purity, the degree of racemization of the glutamic acid residues was found to be less than 2 mol % in all of the fractions listed in Table II.

The concentration dependences of the apparent molecular weights for each fraction, shown in Figures 1 and 2, are not very large. Also, the plot of ln $c$ vs. $r^2$ (where $c$ is the concentration and $r$ is the distance from the center of the rotor) was almost linear from the meniscus to the bottom of the cell in all of the sedimentation equilibrium experiments. These two facts show that the nonideality which might have been expected to occur because of the large size and possible charged state of the copolymer is not significant under the experimental conditions (especially the temperatures and pH) used here. The concentration dependence of the apparent molecular weight was not affected by changing the pH from 1.5 to

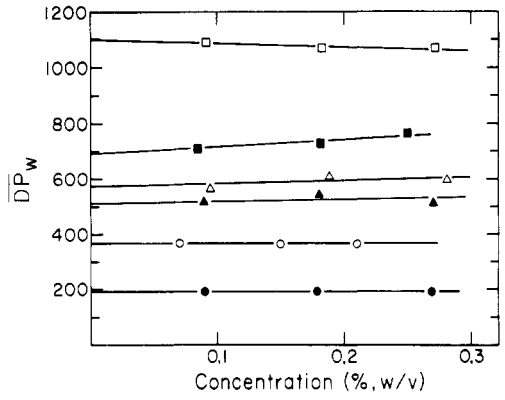


**Figure 1.** Concentration dependence of molecular weights for fractions of L-aspartic acid copolymers used in determining $\sigma$ and $s$: (□) 13.1% Asp, $\overline{DP}_w$ = 1100 (fraction L-II-3); (■) 8.1% Asp, $\overline{DP}_w$ = 690 (fraction L-I-2); (△) 14.2% Asp, $\overline{DP}_w$ = 575 (fraction L-II-5); (▲) 8.3% Asp, $\overline{DP}_w$ = 515 (fraction L-I-5); (○) 13.6% Asp, $\overline{DP}_w$ = 370 (fraction L-II-6); (●) 9.1% Asp, $\overline{DP}_w$ = 190 (fraction L-I-7).

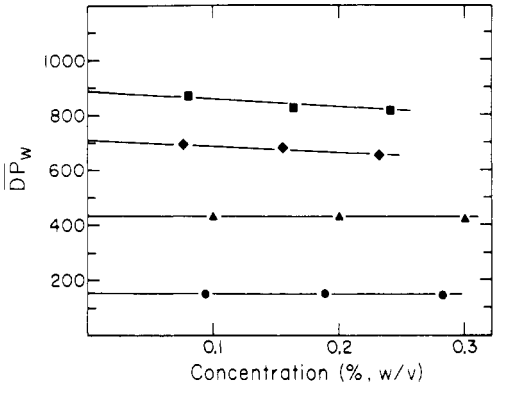


**Figure 2.** Concentration dependence of molecular weights for fractions of D-aspartic acid copolymers used in determining $\sigma$ and $s$: (■) 8.2% Asp, $\overline{DP}_w$ = 890 (fraction D-I-2); (♦) 8.1% Asp, $\overline{DP}_w$ = 710 (fraction D-I-3); (▲) 10.6% Asp, $\overline{DP}_w$ = 430 (fraction D-I-4); (●) 16.9% Asp, $\overline{DP}_w$ = 155 (fraction (D-I-5).

1.0. This suggests that pH 1.5 is low enough to suppress ionization of the carboxyl groups. Over a period of a week, no degradation (i.e., no loss in molecular weight) was detected at pH 1.5 at 25 °C. Table II summarizes the information about the molecular weight and homogeneity of the fractions. The weight-average molecular weights were obtained by extrapolation of the apparent molecular weights to infinite dilution. The $\overline{M}_z/\overline{M}_w$ ratios do not depart significantly from unity and indicate that the fractionation procedure yielded relatively homogeneous materials.

**(B) ORD and CD Data for the Copolymers.** The ORD and CD data for representative fractions of poly(HBG,L-Asp) and poly(HBG,D-Asp) which have different compositions and chain lengths are shown in Figure 3. These measurements were carried out at high and low pH in water and 0.1 N KCl.

Both the ORD and the CD spectra are interpretable in terms of contributions from right-handed $\alpha$-helical and random-coil structures.[50,51] The spectra indicate that even D-aspartic acid residues are incorporated into a right-handed $\alpha$ helix. This is consistent with other experimental results showing that DL copolymers have only one helical screw sense when one of the isomers is present to a very large extent.[49,52] It is also consistent with the results from conformational energy calculations on regular-sequence D,L copolymers.[53] The relative contribution of each conformation is a function of temperature, composition, and pH. With increasing temper-

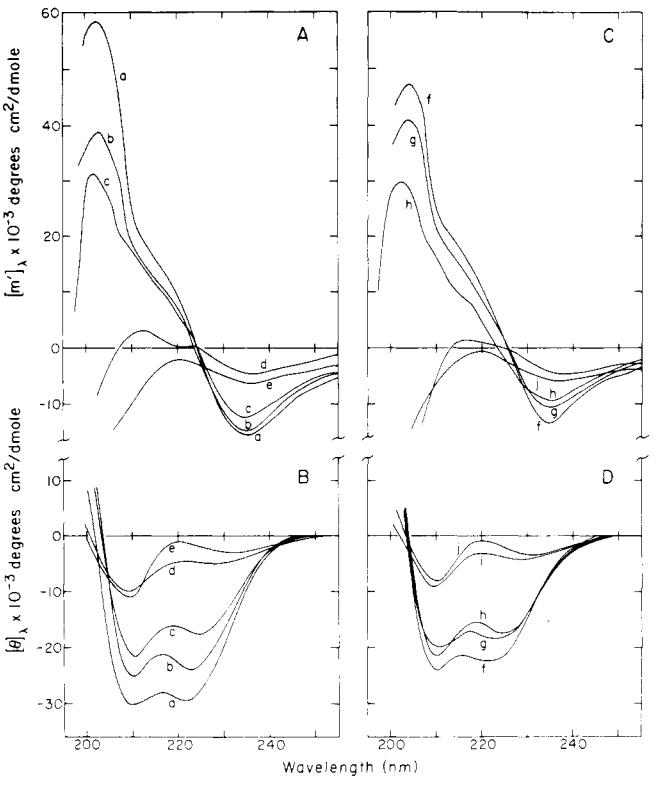


**Figure 3.** (A) ORD and (B) CD data for representative fractions of L-aspartic acid copolymers: (a) 13.1% Asp at pH 1.5, $\overline{DP}_w$ = 1100 (fraction L-II-3) at 2 °C in 0.1 N KCl; (b) 8.3% Asp at pH 8, $\overline{DP}_w$ = 515 (fraction L-I-5) at 2 °C in water; (c) 13.1% Asp at pH 1.5, $\overline{DP}_w$ = 1100 (fraction L-II-3) at 25 °C in water; (d) 13.1% Asp at pH 1.5, $\overline{DP}_w$ = 1100 (fraction L-II-3) at 70 °C in water; (e) 13.6% Asp at pH 8, $\overline{DP}_w$ = 370 (fraction L-II-6) at 50 °C in 0.1 N KCl. (C) ORD and (D) CD data for representative fractions of D-aspartic acid copolymers: (f) 8.2% Asp at pH 1.5, $\overline{DP}_w$ = 890 (fraction (D-I-2) at 10 °C in 0.1 N KCl; (g) 8.1% Asp at pH 1.5, $\overline{DP}_w$ = 710 (fraction D-I-3) at 25 °C in water; (h) 8.2% Asp at pH 8, $\overline{DP}_w$ = 890 (fraction D-I-2) at 2 °C in 0.1 N KCl; (i) 8.1% Asp at pH 1.5, $\overline{DP}_w$ = 710 (fraction D-I-3) at 70 °C in water; (j) 10.6% Asp at pH 8, $\overline{DP}_w$ = 430 (fraction D-I-4) at 50 °C in 0.1 N KCl.

ature and increasing pH, the ORD and CD spectra become characteristic of larger amounts of random coil mixed with smaller amounts of helix. Comparing poly(HBG,L-Asp) and poly(HBG,D-Asp), the former has a larger amount of helix than the latter under any given conditions. These results indicate that these copolymers undergo a thermally and pH induced transition from the $\alpha$ helix to the random coil in these solvents.

The ORD spectra in the range 270–420 nm were used to calculate the values of the Moffitt–Yang parameter $b_0$ at various temperatures, as described previously.[54] The thermally induced helix–coil transition curves were computed from the temperature dependence of $b_0$. Figures 4 and 5 show the transition curves at pH 1.5 and 8 in 0.1 N KCl solution for the ten fractions listed in Table II, and Figures 6 and 7 show the corresponding data at pH 8 in water. The curves exhibited no concentration dependence and were found to be reversible and reproducible. The size of the error symbols in Figures 4–7 indicates two standard deviations in $b_0$ and reflects the errors in the determination of concentration and of the slope of the Moffitt–Yang plot. These errors were estimated to be $\pm 0.030 b_0$ for the former and to be $\pm 3$ (in $b_0$ units) for the latter.

At pH 1.5, the melting curves of all fractions were the same in water and 0.1 N KCl. This means that 0.1 N KCl has no measurable effect on these copolymers at low pH. The low-pH melting curves are shown in Figures 4 and 5, only in 0.1 N KCl. At pH 8, the helix content was found to increase with increasing concentration of salt. It is apparent that there are long-range electrostatic interactions between the charged aspartic acid side chains and that these interactions influence the conformation of the copolymer; also, these interactions are suppressed by salt which shields the charges from one another. No difference could be detected among the melting curves in 0.05, 0.1, and 0.5 N KCl for both fractions L-II-5 and D-I-5 having the highest L-aspartic acid and D-aspartic acid content, respectively. Thus, the shielding effect of the added salt is maximal at less than 0.1 N KCl.

Figures 4 and 6 indicate the following general aspects of the melting curves for poly(HBG,L-Asp). First, the presence of aspartic acid residues decreases the helix content of all fractions relative to that of the homopolymer, poly(HBG), of comparable chain length. Second, for a given aspartic acid content, the helix content increases with increasing chain length. Third, for a given chain length, the helix content decreases with increasing aspartic acid content. These three conclusions apply (roughly) in each solvent system. Fourth, among the different solvent systems, the helix contents (for a given chain length and composition) decrease in the following order: pH 1.5 in 0.1 N KCl (and in water), pH 8 in 0.1 N KCl, pH 8 in water.

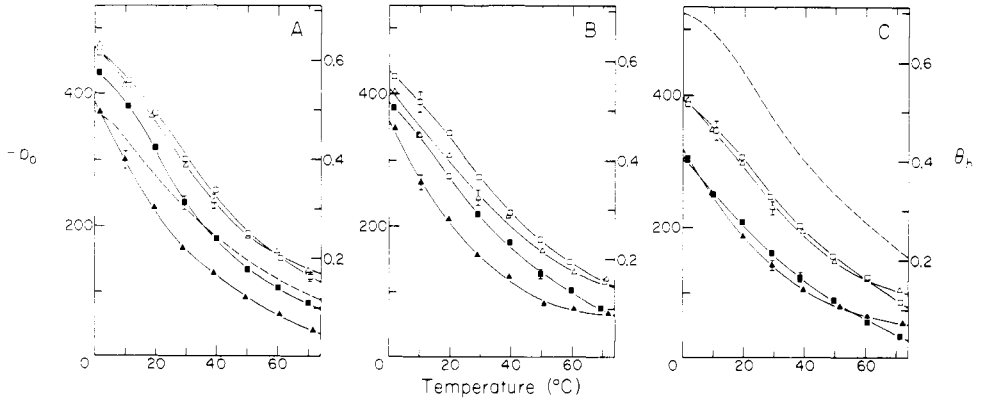


**Figure 4.** Temperature dependence of $b_0$ for poly(HBG,L-Asp) copolymers in 0.1 N KCl at pH 1.5 and 8. Poly(HBG) of $\overline{DP}_w$ = 720 and 200 (fractions V and VIB, respectively, of paper II[15]) are included for comparison: (A) 8.1% Asp, $\overline{DP}_w$ = 690 (fraction L-I-2) at pH 1.5 (□) and pH 8 (■); 13.1% Asp, $\overline{DP}_w$ = 1100 (fraction L-II-3) at pH 1.5 (△) and pH 8 (▲); poly(HBG), $\overline{DP}_w$ = 200 (dashed line); (B) 8.3% Asp, $\overline{DP}_w$ = 515 (fraction L-I-5) at pH 1.5 (□) and pH 8 (■); 14.2% Asp, $\overline{DP}_w$ = 575 (fraction L-II-5) at pH 1.5 (△) and pH 8 (▲); (C) 9.1% Asp, $\overline{DP}_w$ = 190 (fraction L-I-7) at pH 1.5 (□) and pH 8 (■); 13.6% Asp, $\overline{DP}_w$ = 370 (fraction L-II-6) at pH 1.5 (△) and pH 8 (▲); poly(HBG), $\overline{DP}_w$ = 720 (dashed line). The points are the experimental ones, and the lines represent the smoothed experimental curves. The size of the error symbols corresponds to the experimental errors in $\theta_h$ arising from errors in the determination of concentration and in the slope of the Moffitt–Yang plot.

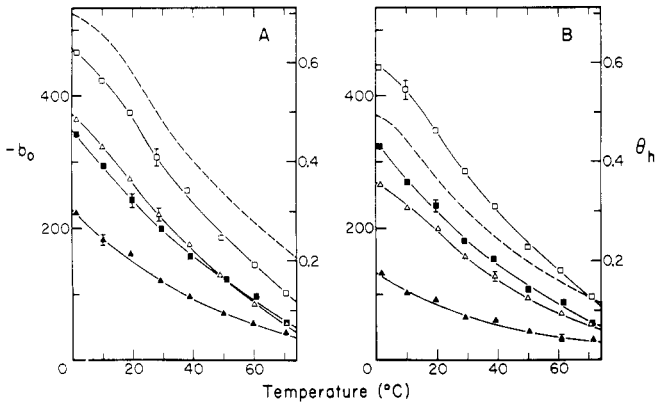


**Figure 5.** Temperature dependence of $b_0$ for poly(HBG,D-Asp) copolymers in 0.1 N KCl at pH 1.5 and 8. Poly(HBG) of $\overline{DP}_w$ = 720 and 200 (fractions V and VIB, respectively, of paper II[15]) are included for comparison: (A) 8.2% Asp, $\overline{DP}_w$ = 890 (fraction D-I-2) at pH 1.5 (□) and pH 8 (■); 10.6% Asp, $\overline{DP}_w$ = 430 (fraction D-I-4) at pH 1.5 (△) and pH 8 (▲); poly(HBG), $\overline{DP}_w$ = 720 (dashed line); (B) 8.1% Asp, $\overline{DP}_w$ = 710 (fraction D-I-3) at pH 1.5 (□) and pH 8 (■); 16.9% Asp, $\overline{DP}_w$ = 155 (fraction D-I-5) at pH 1.5 (△) and pH 8 (▲); poly(HBG), $\overline{DP}_w$ = 200 (dashed line). The points are the experimental ones, and the lines represent the smoothed experimental curves. The error symbols are the same as in Figure 4.

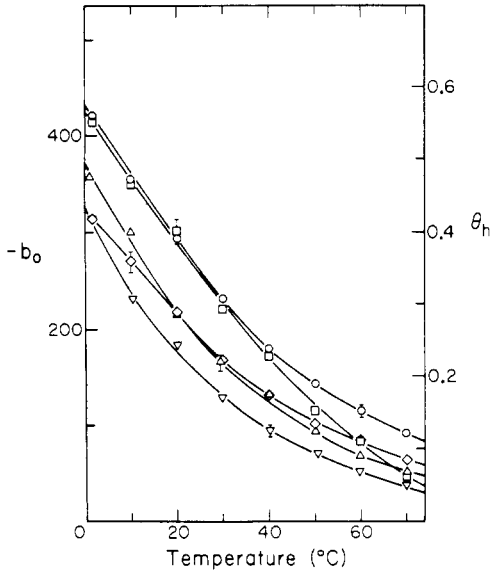


**Figure 6.** Temperature dependence of $b_0$ for poly(HBG,L-Asp) copolymers in water at pH 8: (□) 8.1% Asp, $\overline{DP}_w$ = 690 (fraction L-I-2); (△) 13.1% Asp, $\overline{DP}_w$ = 1100 (fraction L-II-3); (○) 8.3% Asp, $\overline{DP}_w$ = 515 (fraction L-I-5); (▽) 14.2% Asp, $\overline{DP}_w$ = 575 (fraction L-II-5); (◇) 9.1% Asp, $\overline{DP}_w$ = 190 (fraction L-I-7). The points are the experimental ones and the lines represent the smoothed experimental curves. The error symbols are the same as in Figure 4. There was not enough material left to obtain a similar curve for fraction L-II-6.

These facts reveal that the L-aspartic acid residue behaves as a helix breaker and that the charged L-aspartic acid residue is a stronger helix breaker than the uncharged species.

Figures 5 and 7 indicate that these four aspects also pertain to poly(HBG,D-Asp). A comparison between poly(HBG,L-Asp) and poly(HBG,D-Asp) shows that the D-aspartic acid residue is a much stronger helix breaker than the L-aspartic acid residue.

**(C) $b_0$ for the Complete Helix and Complete Coil.** For the homopolymer, poly(HBG), studied in paper II,[15] the value of $b_0$ corresponding to the complete helix was taken to be −750 and the value corresponding to the complete coil was taken to be zero. Because these values vary with the nature of the

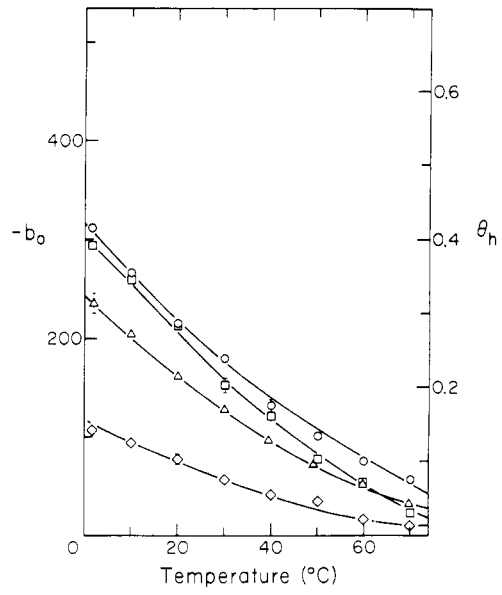


**Figure 7.** Temperature dependence of $b_0$ for poly(HBG,D-Asp) copolymers in water at pH 8: (□) 8.2% Asp, $\overline{DP}_w$ = 890 (fraction D-I-2); (○) 8.1% Asp, $\overline{DP}_w$ = 710 (fraction D-I-3); (△) 10.6% Asp, $\overline{DP}_w$ = 430 (fraction D-I-4); (◇) 16.9% Asp, $\overline{DP}_w$ = 155 (fraction D-I-5). The points are the experimental ones and the lines represent the smoothed experimental curves. The error symbols are the same as in Figure 4.

side chain,[55] several fractions were checked in trifluoroethanol (TFE, a helix-promoting solvent) at 1 °C and in dichloroacetic acid (DCA, a helix-breaking solvent) at 25 °C. After correcting the data for the dispersion of the refractive index of the solvent, typical results were obtained with fraction L-I-2 for which $b_0$ was −739 for the complete helix and with fraction L-II-6 for which $b_0$ was +6 for the complete coil. Similarly, for poly(HBG,D-Asp), $b_0$ was found to be −711 for fraction D-I-3 in TFE and to be −17 for fraction D-I-4 (in DCA). This observation is consistent with previous results which showed that a small amount of D residues incorporated in a polypeptide of L-amino acids does not contribute significantly to the $b_0$ values.[52,56]

These results demonstrate that the assignments of −750 for a complete helix and zero for a complete coil are reasonable ones for analyzing the aspartic acid copolymers, as was done with the other copolymers in this series. Thus, the fractional helix content $\theta_h$ was taken to be $-b_0/750$.

**(D) Titration Data.** The results of the titration experiments with the copolymers and *N*-acetyl-*N'*-methylaspartic acid amide are given in Figure 8, plotted as $pK_{app}$ vs. the degree of ionization, $\alpha$ (where $\alpha$ = 1 when all aspartic acid side chains are charged). The titration data for the copolymers of similar composition were usually well within the experimental error of each other, and thus only representative titration curves are shown.

Similar titration curves of poly(L-aspartic acid), which have been reported by Jacobson[7] and by McDiarmid and Doty,[8] show that it has a much simpler behavior than its homologue, poly(L-glutamic acid),[12,57] and does not exhibit the S-shaped portion at intermediate values of $\alpha$ that is characteristic of a pH-induced helix–coil transition. The copolymers examined in this study also exhibit a simple titration behavior. The $pK_{app}$ at $\alpha$ = 0 for all the copolymers is the same (within experimental error), viz., about 4.6. Figure 8 shows the following aspects of the titration behavior: as the degree of ionization rises, the $pK_{app}$ increases; each of the titration curves has an upward curvature; the change in $pK_{app}$ between $\alpha$ = 0 and $\alpha$ = 1 increases as the aspartic acid content increases. This behavior is similar to that of the copolymers, poly(HBG,L-Glu),

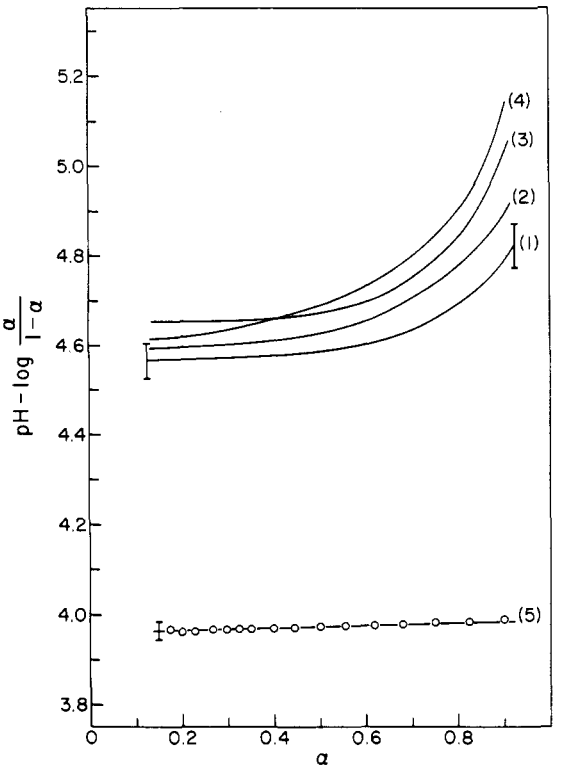


**Figure 8.** A plot of the apparent p$K$ [viz., pH − log $\alpha/(1-\alpha)$] vs. the degree of ionization, $\alpha$ (where $\alpha = 1$ is the fully ionized state), for the copolymers and $N$-acetyl-$N'$-methylaspartic acid amide in 0.1 N KCl at 25 °C: (1) 8.1% L-Asp (fraction L-I-2); (2) 9.1% L-Asp (fraction L-I-7); (3) 10.6% D-Asp (fraction D-I-4); (4) 14.2% L-Asp (fraction L-II-5); (5) $N$-acetyl-$N'$-methylaspartic acid amide.

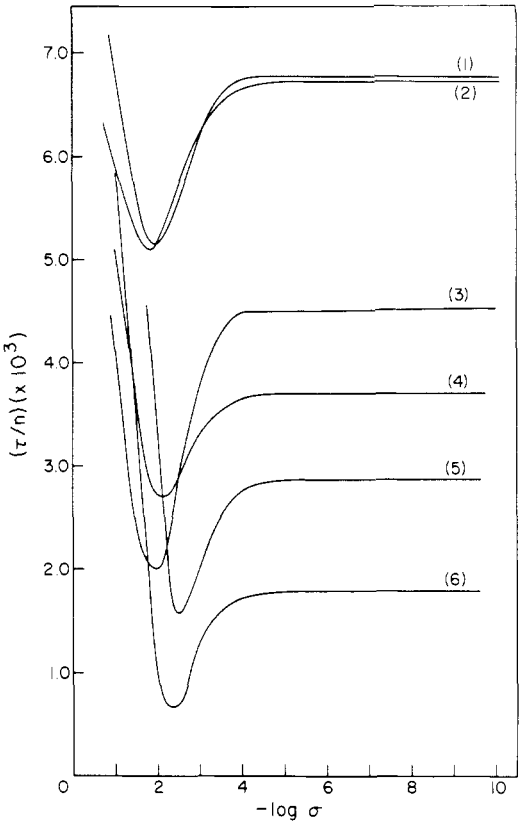


**Figure 9.** Determination of the best temperature-independent value of $\sigma$ as the one which corresponds to the lowest value of $\tau$ for the aspartic acid copolymers: (1) poly(HBG,L-Asp) at pH 1.5 in water; (2) poly(HBG,L-Asp) at pH 8 in 0.1 N KCl; (3) poly(HBG,D-Asp) at pH 8 in water; (4) poly(HBG,L-Asp) at pH 8 in water; (5) poly(HBG,D-Asp) at pH 1.5 in 0.1 N KCl; (6) poly(HBG,D-Asp) at pH 8 in 0.1 N KCl.

investigated in paper IX in this series.[12]

The p$K_a$ of the $N$-acetyl-$N'$-methylaspartic acid amide was found to be 3.96 ± 0.02; this is considerably below the p$K_0$ of the copolymers examined in this study, where p$K_0$ is obtained by extrapolation of p$K_{app}$ to $\alpha = 0$.

The titration curves of all fractions in Table II were reversible, and the numbers of free carboxyl groups were in agreement, within ±3%, with the aspartic acid content determined from amino acid analysis and concentrations.

## III. Discussion

**(A) Helix–Coil Parameters for Poly(L-aspartic acid).** The melting curves described in section II were analyzed according to the LAPS (Lifson–Allegra–Poland–Scheraga) hierarchy of approximations to obtain $\sigma$ and $s$ for poly(aspartic acid). This procedure has been discussed extensively in earlier papers of this series.[15,46,58]

In order to conserve computer time, the first-order approximation, corresponding to the theory of Lifson,[59] was used initially to obtain the values of $\sigma$ and $s$. Then the values were refined with the second-order approximation corresponding to the theory of Allegra.[60] For representative cases, these values were compared with those which were obtained by the exact theory of Lehman and McTague.[61]

The data for poly(HBG,L-Asp) and for poly(HBG,D-Asp) were analyzed separately in the three solvents, pH 1.5 in 0.1 N KCl, pH 8 in 0.1 N KCl, and pH 8 in water.

In all cases, the melting data were analyzed by assuming that $\sigma$ is independent of temperature. The best value of $\sigma$ was obtained by application of the "goodness of fit" criterion defined in paper II[15] and expressed in terms of the parameter $\tau$. The best fit for the data for all fractions in each solvent system was obtained by minimizing $\tau$. Figure 9 shows graphs of $\tau/n$ vs. $\sigma$, where $n$ is the number of samples in each solvent system. The values of $\sigma$ which give the minimum values of $\tau$ are listed in Table III for each solvent system.

**Table III**
**Values of the Zimm–Bragg Parameter $\sigma$ for Poly(aspartic acid) [a]**

| | Solvent conditions | Lifson[b] | Allegra[c] |
|---|---|---|---|
| L-Aspartic acid | pH 1.5, 0.1 N KCl | 0.0166 | 0.0125 |
| | pH 8, 0.1 N KCl | 0.0105 | 0.0050 |
| | pH 8, Water | 0.0070 | 0.0030 |
| D-Aspartic acid | pH 1.5, 0.1 N KCl | 0.0028 | 0.0010 |
| | pH 8, 0.1 N KCl | 0.0052 | 0.00105 |
| | pH 8, Water | 0.0100 | 0.0040 |

[a] The parameters used for poly(HBG) are those of Table II in paper II.[15] [b] The parameters used for aspartic acid were obtained by fitting the data with the Lifson[59] theory. [c] The parameters used for aspartic acid were obtained by fitting the data with the Allegra[60] theory.

The expected helix fractions $(\theta_h)_{theor}$ which were obtained from these calculations[62] are shown (for representative fractions) in Tables IV and V along with the original experimental data $(\theta_h)_{exptl}$ for comparison. Both the first-order (Lifson) and the second-order (Allegra) approximations give results in good agreement with those calculated by the exact method (Lehman–McTague). The higher order (Allegra) approximation will be used in all subsequent discussion of the aspartic acid parameters.

The best value of $s$ at each temperature was found using the Allegra theory with the corresponding values of $\sigma$. They are shown in Tables VI and VII and illustrated in Figures 10, 11,

**Table IV**
**Comparison of the Values of $\theta_h$ Calculated with the Approximate and Exact Theories for Finite Chains [a]**

| L-Aspartic acid content, mol % | $\overline{DP}_w$ | Solvent conditions | Temp, °C | $(\theta_h)_{exptl}$ | $(\theta_h)_{theor}$ Lifson | Allegra | Lehman–McTague[b] |
|---|---|---|---|---|---|---|---|
| 8.1 | 690 | pH 1.5, 0.1 N KCl | 2 | 0.612 | 0.621 | 0.620 | 0.618 |
| | | | 30 | 0.404 | 0.394 | 0.394 | 0.392 |
| | | | 60 | 0.208 | 0.206 | 0.206 | 0.205 |
| 8.1 | 690 | pH 8, 0.1 N KCl | 2 | 0.572 | 0.570 | 0.559 | |
| | | | 30 | 0.317 | 0.296 | 0.291 | |
| | | | 60 | 0.140 | 0.126 | 0.125 | |
| 8.3 | 515 | pH 1.5, 0.1 N KCl | 2 | 0.565 | 0.612 | 0.617 | |
| | | | 30 | 0.365 | 0.390 | 0.394 | |
| | | | 60 | 0.184 | 0.205 | 0.206 | |
| 8.3 | 515 | pH 8, water | 2 | 0.540 | 0.517 | 0.559 | 0.554 |
| | | | 30 | 0.303 | 0.278 | 0.294 | 0.290 |
| | | | 60 | 0.110 | 0.124 | 0.129 | 0.127 |
| 13.1 | 1100 | pH 1.5, 0.1 N KCl | 2 | 0.620 | 0.554 | 0.554 | |
| | | | 30 | 0.386 | 0.342 | 0.341 | |
| | | | 60 | 0.206 | 0.175 | 0.176 | |
| 13.1 | 1100 | pH 8, 0.1 N KCl | 2 | 0.497 | 0.440 | 0.441 | 0.439 |
| | | | 30 | 0.221 | 0.208 | 0.209 | 0.208 |
| | | | 60 | 0.081 | 0.086 | 0.086 | 0.086 |

[a] Footnotes *a–c* and the values of $\sigma$ of Table III apply here. [b] The values of $\sigma$ used in this calculation were those from the Allegra[60] theory.

**Table V**
**Comparison of the Values of $\theta_h$ Calculated with the Approximate and Exact Theories for Finite Chains [a]**

| D-Aspartic acid content, mol % | $\overline{DP}_w$ | Solvent conditions | Temp, °C | $(\theta_h)_{exptl}$ | $(\theta_h)_{theor}$ Lifson | Allegra | Lehman–McTague[b] |
|---|---|---|---|---|---|---|---|
| 8.1 | 710 | pH 1.5, 0.1 N KCl | 2 | 0.420 | 0.442 | 0.436 | |
| | | | 30 | 0.241 | 0.246 | 0.248 | |
| | | | 60 | 0.103 | 0.105 | 0.106 | |
| 10.6 | 430 | pH 8, water | 2 | 0.311 | 0.277 | 0.282 | |
| | | | 30 | 0.168 | 0.153 | 0.154 | |
| | | | 60 | 0.063 | 0.057 | 0.057 | |
| 16.9 | 155 | pH 8, 0.1 N KCl | 2 | 0.163 | 0.149 | 0.153 | 0.149 |
| | | | 30 | 0.094 | 0.102 | 0.096 | 0.096 |
| | | | 60 | 0.046 | 0.046 | 0.043 | 0.043 |

[a] Footnotes *a–c* and the values of $\sigma$ of Table III apply here. [b] The value of $\sigma$ used in this calculation was that from the Allegra[60] theory.

and 12. The error symbols on the computed values of $s$ in these figures were calculated by using the best fit values of $\sigma$; they represent the standard deviations in $s$ at a given temperature. Because the theories used here treat only near-neighbor interactions, and thus do not provide for long-range electrostatic repulsions between the charged side chains,[46] nonrandom errors probably would be included in the deviations for the copolymers at pH 8 in water. Thus, the standard deviations were not estimated in the case of the copolymers at pH 8 in water (Figure 12) where the carboxyl groups of aspartic acid residues are charged.

The values of $s$ calculated for individual fractions were not found to vary in any regular way with composition, implying the absence of specific aspartic acid–aspartic acid interactions in these copolymers.

The melting curves computed with the best-fit Allegra values of $\sigma$ and $s$ at each temperature are shown in Figure 13 for L-aspartic acid in 0.1 N KCl, in Figure 14 for D-aspartic acid in 0.1 N KCl, and in Figures 15 and 16 for pH 8 in water. The experimentally determined points are also shown in these figures. The error symbols here represent errors in the determination of molecular weight (±5%) and amino acid composition (±2.5%). It is apparent that the agreement between the calculated and experimental values of $\theta_h$ is reasonably good in most cases.

The thermodynamic quantities for the conversion of an aspartic acid residue from a coil state to a helical state at the end of a long helical sequence can be obtained from $s$ and its temperature dependence. The free energy ($\Delta G°$) can be obtained directly from $s$ (i.e., $\Delta G° = -RT \ln s$). Figure 17 shows a representative plot of $\Delta G°$ vs. temperature with error symbols calculated from the standard deviations in $s$. The enthalpy ($\Delta H°$) was determined from the slope of the ln $s$ vs. $1/T$ curves at 20 °C, using the van't Hoff equation [$\Delta H° = -R$ d ln $s$/d($1/T$)]. The entropy ($\Delta S°_{20}$) was calculated directly from the relation $\Delta G° = \Delta H° - T\Delta S°$. These thermodynamic quantities are listed in Table VIII. These values should be regarded only as rough estimates in view of the large errors involved in their computation.

Although the discussion of poly(HBG,L-Asp) and poly(HGB,D-Asp) has been carried out separately, *up to here*, the contribution of the significant amount of D-aspartic acid residues in poly(HBG,L-Asp) to the helix–coil stability parameters cannot be neglected. As mentioned in section IIA,

**Table VI**
**Values of the Zimm–Bragg Parameter $s$ for Poly(L-aspartic acid)**

| Temp, °C | $s$[a] | | |
|---|---|---|---|
| | pH 1.5 | pH 8 in 0.1 N KCl | pH 8 in water |
| 2 | 0.82 | 0.71 | 0.71 |
| 10 | 0.80 | 0.69 | 0.69 |
| 20 | 0.78 | 0.66 | 0.67 |
| 30 | 0.74 | 0.63 | 0.65 |
| 40 | 0.71 | 0.59 | 0.62 |
| 50 | 0.68 | 0.54 | 0.59 |
| 60 | 0.63 | 0.49 | 0.53 |
| 70 | 0.60 | 0.45 | 0.47 |

[a] Calculated using the Allegra[60] theory; the values of $\sigma$ used in these calculations are listed in Table III.

**Table VII**
**Values of the Zimm–Bragg Parameter $s$ for Poly(D-aspartic acid)**

| Temp, °C | $s$[a] | | |
|---|---|---|---|
| | pH 1.5 | pH 8 in 0.1 N KCl | pH 8 in water |
| 2 | 0.76 | 0.58 | 0.53 |
| 10 | 0.78 | 0.59 | 0.53 |
| 20 | 0.79 | 0.60 | 0.53 |
| 30 | 0.79 | 0.60 | 0.51 |
| 40 | 0.79 | 0.58 | 0.47 |
| 50 | 0.77 | 0.54 | 0.40 |
| 60 | 0.73 | 0.48 | 0.32 |
| 70 | 0.67 | 0.43 | 0.15 |

[a] Calculated using the Allegra[60] theory; the values of $\sigma$ used in these calculations are listed in Table III.

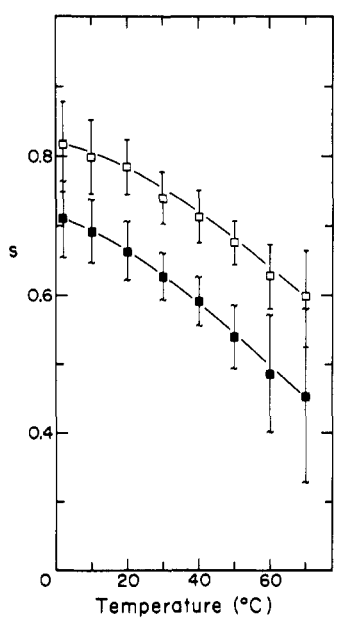


**Figure 10.** A plot of $s$ vs. $T$ for poly(L-aspartic acid) in 0.1 N KCl at pH 1.5 (□) and at pH 8 (■). The solid lines are drawn to pass through all the points. The error symbols are described in section IIIA.

we could not synthesize poly(HBG,L-Asp) without involving simultaneously both racemization and transamidation of the L-aspartic acid residues. We, therefore, chose to work with samples of poly(HBG,L-Asp) which contain about 15% of D-aspartic acid residues but which are free from $\beta$-peptide linkages.

If we make an assumption, we can obtain the helix–coil stability parameters for *pure* L- and D-aspartic acids by

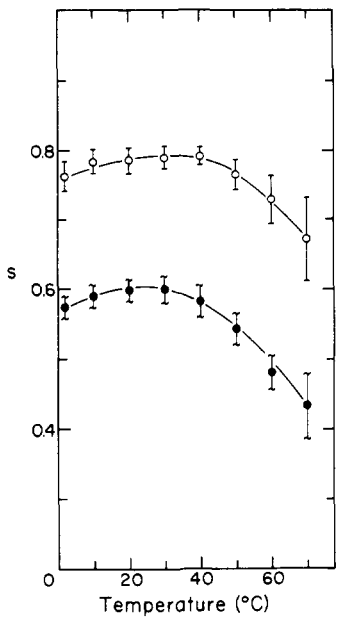


**Figure 11.** A plot of $s$ vs. $T$ for poly(D-aspartic acid) in 0.1 N KCl at pH 1.5 (○) and at pH 8 (●). The solid lines are drawn to pass through all the points. The error symbols are described in section IIIA.

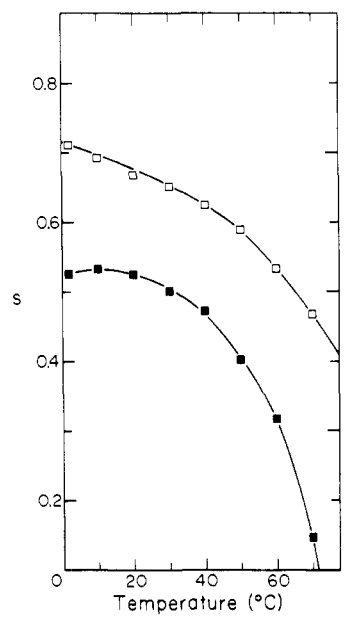


**Figure 12.** A plot of $s$ vs. $T$ for poly(aspartic acid) in water at pH 8: poly(L-aspartic acid) (□) and poly(D-aspartic acid) (■). See text for omission of error symbols.

eliminating the effect of racemization (in the calculations).

The assumption is that the apparent free-energy changes which were determined experimentally with poly(HBG,L-Asp) and poly(HBG,D-Asp) reflect a linear combination of intrinsic free energy changes for *pure* L- and D-aspartic acids. This hypothesis should provide a good enough approximation for a first-order estimation of the effect of racemization on $\sigma$ and $s$. Then, the apparent free-energy changes for L- and D-aspartic acid, $\Delta G°_{L,app}$ and $\Delta G°_{D,app}$, which were determined with poly(HGB,L-Asp) and poly(HBG,D-Asp), respectively, can be expressed as:

$$\Delta G°_{L,app} = (1 - m_D)\Delta G°_L + m_D \Delta G°_D \quad (2)$$

$$\Delta G°_{D,app} = (1 - m_L)\Delta G°_D + m_L \Delta G°_L \quad (3)$$

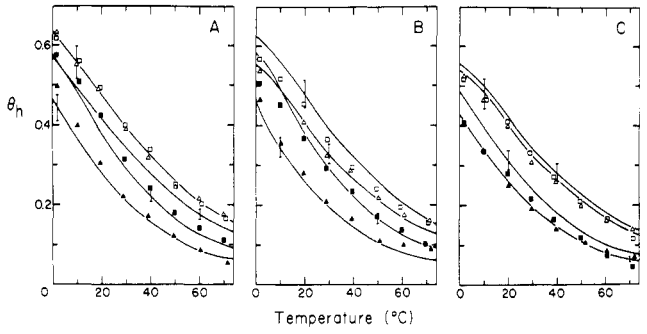


**Figure 13.** Comparison of the calculated melting curves, obtained from the parameters of the Allegra theory for L-aspartic acid given in Tables III and VI and those of poly(HBG) of Table II in paper II,[15] with the experimental points for copolymers in 0.1 N KCl: (A) 8.1% Asp, $\overline{DP}_w$ = 690 (fraction L-I-2) at pH 1.5 (□) and pH 8 (■); 13.1% Asp, $\overline{DP}_w$ = 1100 (fraction L-II-3) at pH 1.5 (△) and pH 8 (▲); (B) 8.3% Asp, $\overline{DP}_w$ = 515 (fraction L-I-5) and pH 1.5 (□) and pH 8 (■); 14.2% Asp, $\overline{DP}_w$ = 575 (fraction L-II-5) at pH 1.5 (△) and pH 8 (▲); (C) 9.1% Asp, $\overline{DP}_w$ = 190 (fraction L-I-7) at pH 1.5 (□) and pH 8 (■); 13.6% Asp, $\overline{DP}_w$ = 370 (fraction L-II-6) at pH 1.5 (△) and pH 8 (▲). The error symbols indicate errors in the calculated values of $\theta_h$ arising from errors in composition and chain length (see Figure 4 for additional errors in experimental points).

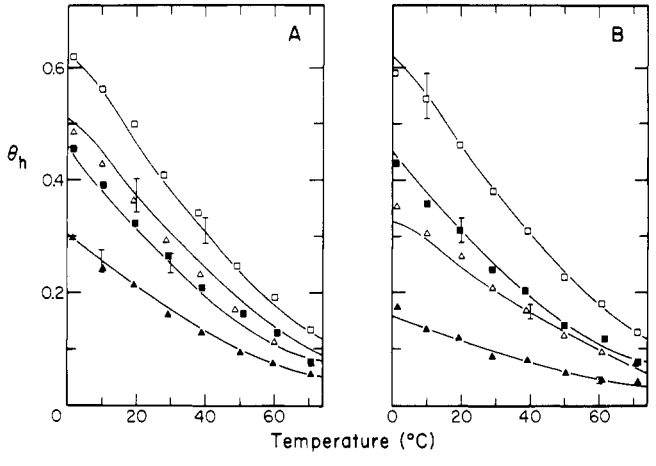


**Figure 14.** Comparison of the calculated melting curves, obtained from the parameters of the Allegra theory for D-aspartic acid given in Tables III and VII and those of poly(HBG) of Table II in paper II,[15] with the experimental points for copolymers in 0.1 N KCl: (A) 8.2% Asp, $\overline{DP}_w$ = 890 (fraction D-I-2) at pH 1.5 (□) and pH 8 (■); 10.6% Asp, $\overline{DP}_w$ = 430 (fraction D-I-4) at pH 1.5 (△) and pH 8 (▲); (B) 8.1% Asp, $\overline{DP}_w$ = 710 (fraction D-I-3) at pH 1.5 (□) and pH 8 (■); 16.9% Asp, $\overline{DP}_w$ = 155 (fraction D-I-5) at pH 1.5 (△) and pH 8 (▲). The error symbols indicate errors in the calculated values of $\theta_h$ arising from errors in composition and chain length (see Figure 5 for additional errors in experimental points).

where $\Delta G°_L$ and $\Delta G°_D$ are free-energy changes for *pure* L- and D-aspartic acids, $m_D$ is the mole fraction of D-aspartic acid residues resulting from racemization in poly(HBG,L-Asp), and $m_L$ is the mole fraction of L-aspartic acid residues resulting from racemization in poly(HBG,D-Asp).

Using the relation $\Delta G° = -RT \ln s$, the following equations for the values of $s$ for *pure* L- and D-aspartic acids, $s_L$ and $s_D$, respectively, are derived:

$$\ln s_{L,app} = (1 - m_D) \ln s_L + m_D \ln s_D \quad (4)$$

$$\ln s_{D,app} = (1 - m_L) \ln s_D + m_L \ln s_L \quad (5)$$

The average values of $m_L$ and $m_D$ were estimated roughly to be 0.15 from the data listed in Table II. This estimation is not unreasonable, in the spirit of the accuracy of the following discussion. In calculating the values of $s$ for *pure* L and D, the data for $s$ listed in Tables VI and VII were used for $s_{L,app}$ and $s_{D,app}$, respectively. Equations 4 and 5 were then solved simultaneously to obtain $s_L$ and $s_D$ at each temperature. The values of $s$ for *pure* L-aspartic acid in the charged and un-

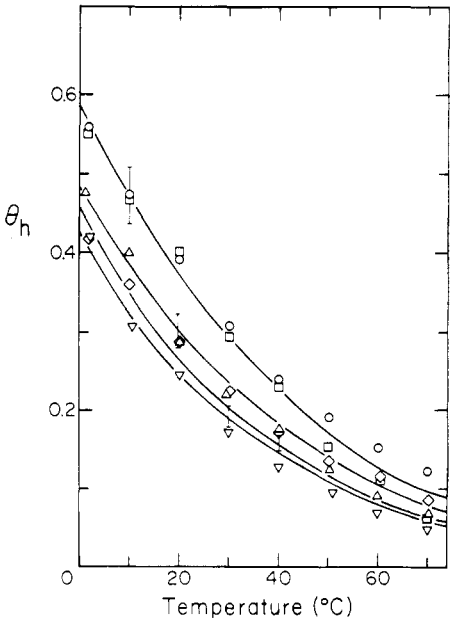


**Figure 15.** Comparison of the calculated melting curves, obtained from the parameters of the Allegra theory for L-aspartic acid given in Tables III and VI and those of poly(HBG) of Table II in paper II,[15] with the experimental points for copolymers in water at pH 8: (□) 8.1% Asp, $\overline{DP}_w$ = 690 (fraction L-I-2); (△) 13.1% Asp, $\overline{DP}_w$ = 1100 (fraction L-II-3); (○) 8.3% Asp, $\overline{DP}_w$ = 515 (fraction L-I-5); (▽) 14.2% Asp, $\overline{DP}_w$ = 575 (fraction L-II-5); (◇) 9.1% Asp, $\overline{DP}_w$ = 190 (fraction L-I-7). The error symbols indicate errors in the calculated values of $\theta_h$ arising from errors in composition and chain length. The curves for □ and ○ lie close to each other, and only one is shown (see Figure 6 for additional errors in experimental points).

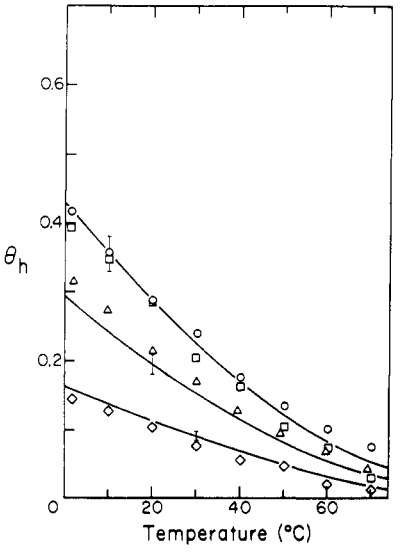


**Figure 16.** Comparison of the calculated melting curves, obtained from the parameters of the Allegra theory for D-aspartic acid given in Tables III and VII and those of poly(HBG) of Table II in paper II,[15] with the experimental points for copolymers in water at pH 8: (□) 8.2% Asp, $\overline{DP}_w$ = 890 (fraction D-I-2); (○) 8.1% Asp, $\overline{DP}_w$ = 710 (fraction D-I-3); (△) 10.6% Asp, $\overline{DP}_w$ = 430 (fraction D-I-4); (◇) 16.9% Asp, $\overline{DP}_w$ = 155 (fraction D-I-5). The error symbols indicate errors in the calculated values of $\theta_h$ arising from errors in composition and chain length. The curves for □ and ○ lie close to each other, and only one is shown (see Figure 7 for additional errors in experimental points).

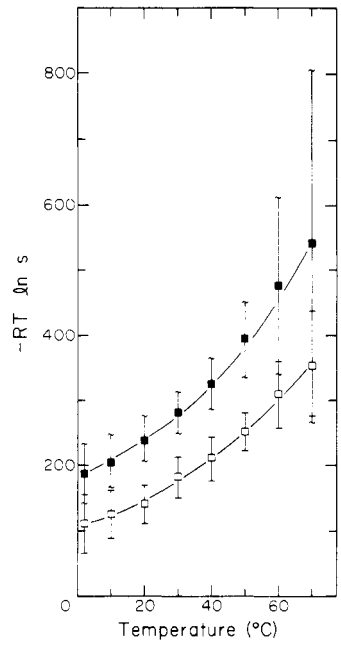


**Figure 17.** A plot of $-RT \ln s$ (i.e., $\Delta G°$) vs. $T$ for poly(L-aspartic acid) at pH 1.5 (□) and at pH 8 (■) in 0.1 N KCl. The error symbols are described in section IIIA.

**Table VIII**
**Thermodynamic Parameters for L-Aspartic Acid**

| | pH 1.5 | pH 8 in 0.1 N KCl |
|---|---|---|
| $\Delta G°_{20}$, cal/mol | 141 | 239 |
| $\Delta H°_{20}$, cal/mol | −1025 | −1300 |
| $\Delta S°_{20}$, eu | −3.98 | −5.25 |

charged states were calculated at each temperature using the data at pH 1.5 and 8 in 0.1 N KCl, respectively, and are listed in Table IX.

The values of $\sigma_L$ and $\sigma_D$ for *pure* L- and D-aspartic acids were calculated in the same way, using the following equations

$$\ln \sigma_{L,app} = (1 - m_D) \ln \sigma_L + m_D \ln \sigma_D \quad (6)$$

$$\ln \sigma_{D,app} = (1 - m_L) \ln \sigma_D + m_L \ln \sigma_L \quad (7)$$

where $\sigma_{L,app}$ and $\sigma_{D,app}$ are the values listed in Table III. The values of $\sigma$ for *pure* L-aspartic acid in the charged and uncharged states are also listed in Table IX.

Comparison between Tables VI and IX shows clearly that the difference between the experimental data for $s$ for L-aspartic acid and the calculated values for *pure* L-aspartic acid is quite small. This means that the contamination of the copolymer by D-aspartic acid residues does not affect the determination of the helix–coil stability parameters for L-aspartic acid very much.

**(B) Titrations.** The titration behavior of poly(HBG,L-Asp) (in Figure 8) is similar to that of poly(HBG,L-Glu)[12] in several respects. For both sets of copolymers, the curves lack the S-shaped character (at intermediate values of $\alpha$) that is characteristic of the pH-induced helix–coil transition in poly(L-glutamic acid). The values of $pK_{app}$ increase with increasing $\alpha$ and with increasing aspartic acid content, and the values of $pK_0$ are higher in the copolymers than in the corresponding model compounds (the *N*-acetyl-*N'*-methylamide derivatives).

In the case of poly(L-glutamic acid) the slope at low $\alpha$ (where the polymer is helical) is greater than that at high $\alpha$ (where it is in the coil form); i.e., the electrostatic free energy of the helix is higher than that of the coil form, causing a helix–coil transition as $\alpha$ increases. In the curves of Figure 8, however, the slopes of the copolymer curves appear to be higher at high $\alpha$; we attribute this to the large experimental error in these titrations and conclude that there is no large difference between the electrostatic free energies of the helical and coil states of the copolymers in 0.1 N KCl, as was observed[12] for poly(HBG,L-Glu). The absence of a salt effect on the melting curves (in the range between 0.05 and 0.5 N KCl) and the similar values of $\sigma$ and $s$ (Tables III and VI) for aspartic acid at pH 8 in water and in 0.1 N KCl (computed from a theory based only on short-range interactions) support this conclusion.

**Table IX**
**Values of the Zimm–Bragg Parameter $s$ for *Pure* L-Aspartic Acid [a]**

| Temp, °C | $s$ | |
|---|---|---|
| | pH 1.5[b] | pH 8[c] in 0.1 N KCl |
| 2 | 0.83 | 0.74 |
| 10 | 0.79 | 0.72 |
| 20 | 0.78 | 0.68 |
| 30 | 0.73 | 0.63 |
| 40 | 0.70 | 0.59 |
| 50 | 0.66 | 0.54 |
| 60 | 0.61 | 0.49 |
| 70 | 0.58 | 0.46 |

[a] See text. [b] $\sigma = 0.021$ (calculated using the Allegra values in Table III). [c] $\sigma = 0.0070$ (calculated using the Allegra values in Table III).

Since the electrostatic free energy of the charged helix and the charged coil are similar in the copolymers used in this study (i.e., those containing less than 17% aspartic acid), the only way that a pH-induced helix–coil transition can occur is for $\sigma$ and $s$ to differ for charged and uncharged side chains, as is the case for poly(HBG,L-Glu);[12] such differences arise from short-range interactions (between the side chain and its own backbone). The reason for the absence of a pH-induced helix–coil transition in poly(L-aspartic acid)[8] is that the low values of $s$ for uncharged Asp (see Table IX) do not permit the formation of helix even when all of the aspartic acid residues are uncharged. For poly(L-glutamic acid), the large value of $s$ for uncharged glutamic acid[12] results in the formation of helix at low pH. As the pH is raised, poly(L-glutamic acid) undergoes a helix–coil transition because of the lower values of $s$ for charged glutamic acid and the effects of electrostatic repulsions between the charged side chains.

As was discussed[12] for poly(HBG,L-Glu), we attribute the increase in $pK_0$ of the side chain carboxyl groups of the copolymers over that of the corresponding *N*-acetyl-*N'*-methylamide derivative (viz., ~4.6 compared to 3.96 ± 0.2) to environmental effects in the copolymers (primarily the nonpolar character of the HBG side chains).

Finally, we note the difference in $pK_0$'s between the side chain aspartic acid and glutamic acid carboxyl groups (3.96 vs. 4.42) in molecules which contain no other ionizable groups. Such a difference also exists for the side-chain carboxyl groups of the free amino acids (3.65 vs. 4.25).[63] Momany et al.[64] reported the CNDO/2 partial charges on all atoms of the *N*-acetyl-*N'*-methylamides of charged and uncharged aspartic acid and glutamic acid. From these data [in which the atoms of the uncharged carboxyl groups of both compounds (and similarly of the charged groups) have the same charges], we find no obvious reason for this difference in $pK_0$'s. Perhaps the formation of a six-membered ring, involving a hydrogen bond between the aspartyl NH and $COO^-$ groups, increases the acidity of the carboxyl group. This explanation may also

**Table X**
**Helix–Coil Stability Parameters for Gly, Ser, Asn, and Asp at 20 °C in Water as Determined by the Host–Guest Technique**

| Amino acid | $\sigma$ | $s$ | $\Delta G°$, cal/mol | $\Delta H°$, cal/mol | $\Delta S°$, eu |
|---|---|---|---|---|---|
| Gly[a] | $1 \times 10^{-5}$ | 0.59 | 305 | 625 | 1.0 |
| Ser[b] | $1 \times 10^{-5}$ | 0.76 | 157 | −305 | −1.6 |
| Asn[c] | $9.5 \times 10^{-6}$ | 0.78 | 142 | 400 | 0.88 |
| Asp[d] (charged[e]) | $7.0 \times 10^{-3}$ | 0.68 | 225 | −1140 | −4.7 |
| Asp[d] (uncharged) | $2.1 \times 10^{-2}$ | 0.78 | 145 | −970 | −3.8 |

[a] Reference 58. [b] Reference 66. [c] Reference 65. [d] This paper. These are the computed values for *pure* L-aspartic acid. [e] In 0.1 N KCl.

account for the fact that Asp racemizes easily whereas Asn[65] and Glu[12] do not.

**(C) Comparison with Other Results.** Previous investigations cited in the introductory section provided only qualitative information about the helix–coil transition in poly(L-aspartic acid) and no information about the differences in conformational preferences of charged and uncharged L-aspartic acid residues. The application of the "host–guest" technique here has revealed that L-aspartic acid is a pronounced helix breaker in water over the temperature range 0–70 °C and that the charged L-aspartic acid residue is a somewhat stronger breaker than the uncharged one. In this series, in which the "host–guest" technique has been used to determine $\sigma$ and $s$, thus far glycine, serine, asparagine, and aspartic acid have been reported to be helix breakers. The helix–coil stability parameters of these helix breakers are listed in Table X. Although the thermodynamic parameters for the coil–helix conversion are different, it is of interest that the values of $s$ for serine, asparagine, and uncharged aspartic acid are almost the same at 20 °C. The value of $s$ for charged Asp is less than that of Asn at 20 °C. Thus, deamidation of Asn to form Asp could destabilize a helical region that included an Asn residue. Possible biological effects of such deamidation were discussed previously.[65]

Several studies of the frequencies of occurrence of amino acids in various conformational states in proteins of known crystal structure have been reported,[67-75] and aspartic acid has been assigned as an indifferent helix maker. Our results indicate, however, that charged aspartic acid, which is the predominant form at physiological pH, is a very strong helix breaker. Such a discrepancy between the classification of amino acids in proteins and in host–guest copolymers, respectively, in terms of their helix-making ability was reported previously for the case of glutamic acid.[12] Maxfield and Scheraga[76] resolved this discrepancy by showing that a positively charged residue frequently was present four residues away from a glutamic acid residue in a protein, thereby greatly enhancing its probability for being in a helical conformation. They also showed that the same type of effect exists in the case of aspartic acid. Thus, the helix–coil stability constants of aspartic acid obtained here provide information about its *intrinsic* tendency for being helical, based on short-range interactions.

## IV. Conclusions

Water-soluble random copolymers containing L-aspartic acid and $N^5$-(4-hydroxybutyl)-L-glutamine were synthesized and characterized. From an analysis of the thermally induced helix–coil transition of these copolymers, the Zimm–Bragg parameters $\sigma$ and $s$ were determined. Based on the value of $s$, L-aspartic acid was shown to be a helix breaker at all temperatures in the range of 0–70 °C.

**Acknowledgment.** We are indebted to H. Chan and T. Thannhauser for performing the nitrogen and amino acid analyses, to G. Davenport for excellent technical assistance, to E. R. Stimson for providing a sample of $N$-acetyl-$N'$-methylaspartic acid amide, and to M. J. Miller and G. M. Loudon for analyzing the copolymers for $\beta$ linkages. We also thank J. E. Alter and G. T. Taylor for carrying out a preliminary investigation of this problem and F. R. Maxfield, R. K. Scheule, and R. R. Matheson for helpful discussions.

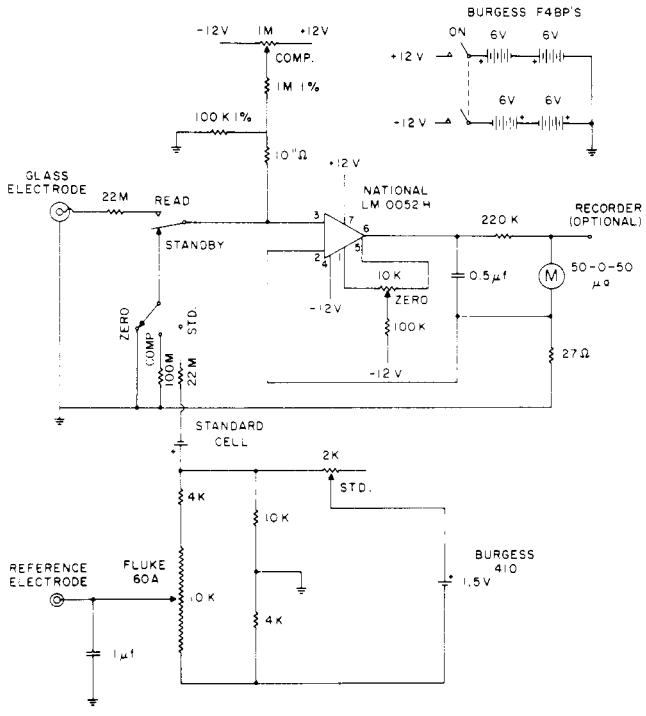


**Figure 18.** Circuit diagram of pH meter.

## Appendix. pH Meter

The pH meter was designed and built in this laboratory by Gary Davenport, using some of the basic components of the instrument described in ref 36. However, most of the electrical circuits were simplified greatly and improved by making use of more recent electronic developments.

The measuring circuit uses a Fluke decade potentiometer, type 60A, 10K Ω as in the previous instrument.[36] However, it was wired to cover both positive and negative voltages in a single full range to avoid switching ranges. Therefore, the dial readings (though related to millivolts) are arbitrary. They are converted to pH units by using a calibration curve relating the pH's of standard buffers to dial readings.

The null detector circuit uses a FET operational amplifier (National LM0052H). This integrated circuit has its dual FET inputs interwoven to achieve a close match of input characteristics. The incredibly low drift rate of the meter (<0.00025 pH units/h) is due mainly to the low input current (<1 pA) and to the bias current compensation circuit incorporated in the meter (see Figure 18). This circuit ensures that minimum

current flows through the electrode system at null condition to avoid polarization in the solution whose pH is being measured.

Batteries are used throughout. The 1.5-V battery of the potentiometer is left connected permanently to improve stability. At this low current drain, it will operate continuously for many hundreds of hours. The condition of the amplifier batteries is indicated by the ability of the null meter to deflect fully to the right or left.

An Ingold Electrodes Inc. combination glass/reference electrode No. 6030-02 was found to work satisfactorily with the meter, exhibiting minimum interference from fluctuations in temperature and electrostatic and magnetic fields (the latter arising from the magnetic stirrer). At low temperatures (approaching 0 °C), some amplifier instability arises from increased electrode impedance and manifests itself as a small oscillation of the null meter. However, it was still possible to obtain readings at 0 °C, but with less accuracy than at room temperature.

To operate the instrument, the null meter is first zeroed with the Zero–Compensate–Standardize switch, making corresponding Zero, Compensate, and Standardize adjustments, in that order. The Read switch is then activated, and the potentiometer nulled. The precision of the reading is ±0.0002 pH units at room temperature.

## References and Notes


(1) This work was supported by research grants from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, U.S. Public Health Service (AM-08465), and from the National Science Foundation (PCM75-08691).
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