Nuclear Magnetic Resonance Studies of Side-Chain Interactions in Polyamino Acids with Aromatic Groups. Comparison to Conformational Energy Calculations*

David N. Silverman† and Harold A. Scheraga‡

ABSTRACT: Nuclear magnetic resonance measurements have been made for poly(γ-benzyl-L-glutamate), poly(γ-methyl-L-glutamate), poly(β-benzyl-L-aspartate), and poly(L-phenylalanine) in the helical and random coil forms in $CDCl_3$–trifluoroacetic acid mixtures. Changes in the chemical shift for side-chain protons during the helix–coil transition $\Delta\sigma_{h\rightarrow c}$ are accounted for in terms of solvent effects and side-chain-side-chain interactions. In particular, the contributions to $\Delta\sigma_{h\rightarrow c}$ arising from the anisotropic magnetic susceptibility of the side-chain aromatic groups are obtained. From these quantities, it is determined that the side chains of helical poly(γ-benzyl-L-glutamate) in $CDCl_3$–trifluoroacetic acid solvents tend to be extended into solution rather than wrapped around the helical backbone. In addition, it is postulated from evidence of phenyl ring stacking (deduced from the values of $\Delta\sigma_{h\rightarrow c}$ for the benzyl group protons) that these extended side chains constitute a secondary side-chain helix in α-helical poly(γ-benzyl-L-glutamate). No evidence is found for a similar ring stacking in the side chains of poly(β-benzyl-L-aspartate). The nuclear magnetic resonance spectra of poly(L-phenylalanine) are also interpreted in terms of side-chain–side-chain interactions. The ring current effect from neighboring aromatic residues is computed for several conformations of the side chains in the helical form of this polymer and compared to the experimental values of the chemical shifts, enabling deductions to be made about the orientations of the side chains. Since a large range of side-chain conformations is consistent with the observed chemical shifts, it is shown that conformational energy calculations can be used to narrow the possible range of conformations for the side chains.

While the importance of the side chains in determining the stability of the α-helical conformation of synthetic homopolyamino acids has been well established (Fasman, 1967), very little information is available from X-ray and optical studies about the orientations of the atoms in the side chains relative to those of the backbone. However, high-resolution nuclear magnetic resonance spectroscopy has recently been applied to this problem,[1] since chemical shifts and line widths provide information about the environment and mobility, respectively, of the side chain (Joubert *et al.*, 1970; Bradbury *et al.*, 1970).

In the present study, nuclear magnetic resonance was used to investigate the orientations of the side chains in the α-helical forms of several homopolyamino acids. While a great deal of work has been done to determine the cause of the changes in the chemical shift of the α-CH proton during the helix–coil transition [Bradbury *et al.*, 1968, 1969; Ferretti and Paolillo, 1969; Joubert *et al.*, 1970], relatively little is known about the chemical shifts of the side-chain protons or about the insight they might provide into the role of the side chains in stabilizing the α-helical conformation. For this purpose, amino acids with side chains containing aromatic groups [*viz.*, poly(γ-benzyl-L-glutamate) $(BzGlu)_n$, poly(β-benzyl-L-aspartate) $(BzAsp)_n$, and poly(L-phenylalanine) (Phe_n)] were chosen in this initial study. These are excellent polymers for a nuclear magnetic resonance study of side-chain interactions, since the effects from ring currents are readily observed as changes in the chemical shift (Pople *et al.*, 1959); also conformational energy calculations, with which the nuclear magnetic resonance results can be compared, have been carried out for these polymers (Yan *et al.*, 1968).

Experimental Section

Materials. Poly(γ-benzyl-L-glutamate) (mol wt ~50,000) was prepared[2] by polymerization of γ-benzyl-L-glutamate *N*-carboxyanhydride initiated with CH_3ONa, and its molecular weight was determined by viscosity. The other samples, together with the suppliers estimate of the molecular weight, were poly(γ-methyl-L-glutamate) (Pilot, ~275,000), poly(β-benzyl-L-aspartate) (Pilot, ~235,000), and poly(L-phenylalanine) (Mann, >100,000); the commercial samples were used without further purification. High molecular weight polymers were chosen in order to obtain a sharp solvent-induced helix–coil transition. By observing this transition over a small range of solvent composition, we minimized the effect of variations in solvent composition on the chemical shift.

Methods. All spectra were obtained on a Varian HA-100 nuclear magnetic resonance spectrometer at room temperature (28° ± 1°) unless otherwise specified. The temperatures reported are accurate to ±1°. Chemical shifts are given in parts per million or in Hz from the internal standard tetra-

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† Postdoctoral Fellow of the National Institute of General Medical Sciences, National Institutes of Health, 1969–1971.

‡ To whom correspondence should be addressed.

[1] Measurements of electric dichroism (Troxell and Scheraga, 1969) and of dipole moments (Erenrich and Scheraga, manuscript in preparation) can also provide information about the relative orientations of the side chains of an α helix.

[2] We are indebted to Patricia H. Von Dreele for the preparation of this sample.

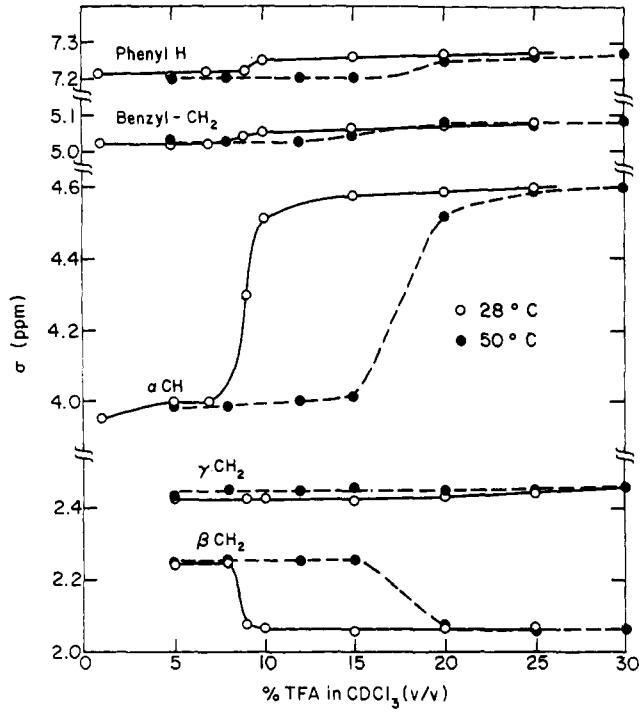

FIGURE 1: Chemical shifts of poly(γ-benzyl-L-glutamate) $(BzGlu)_n$, at 0.07–0.1 M, in solutions of different concentrations of trifluoroacetic acid and $CDCl_3$, at 28°. Values of the chemical shift (σ) are given in ppm from the internal reference Me_4Si. The dotted curve represents the chemical shifts of the same solutions at 50°.

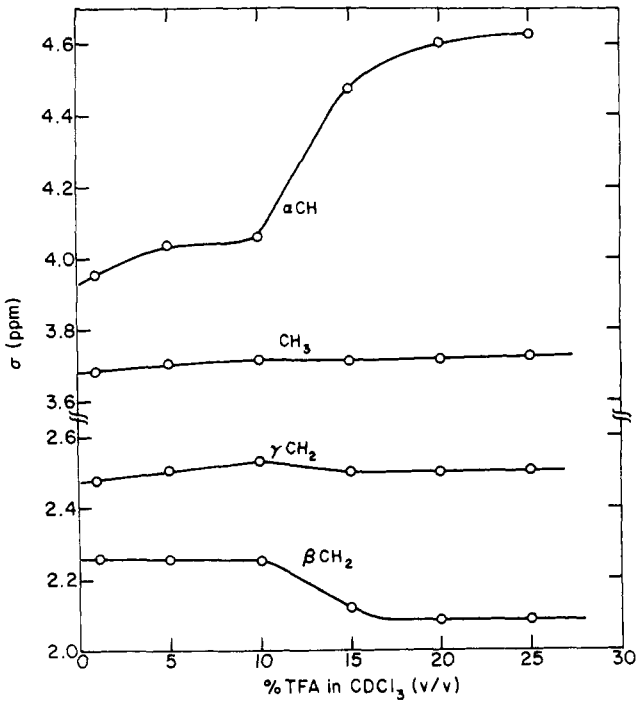

FIGURE 2: Chemical shifts of poly(γ-methyl-L-glutamate) $(MeGlu)_n$, at 0.07–0.1 M, in solutions of different concentrations of trifluoroacetic acid and $CDCl_3$, at 50°. Values of the chemical shifts (σ) are given in ppm from the internal reference Me_4Si.

methylsilane. Each value represents an average of at least five sweeps, and the errors given represent the standard deviation of this number of sweeps. The larger standard deviations arise from the large line widths obtained for polymers in the α-helical conformation.

Double irradiation techniques were used, since they are of considerable help in observing the resonance of α-CH and β-CH_2 protons, as noted by Bradbury *et al.* (1969). Thus, measurement of the α-CH proton peaks in helical polyamino acids was facilitated by simultaneous irradiation at the β-CH_2 proton resonance frequencies. Similarly, β-CH_2 peaks could be sharpened somewhat by strong irradiation at the rather broad α-CH proton resonance.

The concentration of the polymers in solutions of deuterated chloroform ($CDCl_3$) and deuterated trifluoroacetic acid was between 0.07 M and 0.1 M [approximately 1.0–2.2% (w/v)]. All spectra were measured within 2 hr of sample preparation, during which time the helix content remained unchanged. The advantage of using deuterated trifluoroacetic acid is that deuterium ions exchange with backbone NH protons of the polyamino acid in the random coil form; this removes a source of splitting at the α-CH position and sharpens this rather broad peak. The disadvantage is that the backbone NH peak is rendered unobservable. We now must ask how this deuterium exchange affects the helix–coil transition. It has been shown (Elliott and Hanby, 1958) that deuterium exchange of the peptide-group hydrogen atoms occurs very slowly when these hydrogen atoms are participating in the hydrogen bonding of an α helix. The measurements of Elliott and Hanby (1958) were made on the NH-stretching band at 3300 cm^{-1} in a study of $(BzGlu)_n$ in organic solvents and showed no large degree of exchange in less than a day. In our study, measurements of the helix–coil transition were performed on polymers that were protonated in the helical form. Consequently, the possible effect of a D–H substitution on the strength of N–H . . . O hydrogen bonds (Calvin *et al.*, 1959; Tomita *et al.*, 1962), and hence on the stability of the helix, does not influence the results reported here since the deuterium exchange is very slow in the helical conformation.

Results

A. Poly(γ-benzyl-L-glutamate). The chemical shifts of the various protons of $(BzGlu)_n$ as a function of solvent composition at 28° are shown in Figure 1. These data are in agreement with recently published results on $(BzGlu)_n$ (Bovey, 1968). The large changes observed at approximately 8–14% trifluoroacetic acid are caused by the transition from a right-handed α helix to a random coil. The chemical shifts of the same protons at 50° are shown as a dotted curve in Figure 1. Because of the inverse thermal transition (Doty and Yang, 1956), the solvent-induced helix–coil transition at 50° occurs at approximately 15–20% trifluoroacetic acid. For comparison, the chemical shifts of poly(γ-methyl-L-glutamate) $(MeGlu)_n$ at 50° are shown in Figure 2; spectra could not be obtained for this polymer at 28° because it is not sufficiently soluble in the range of solvent compositions of interest. Examination of Figures 1 and 2 and the data of Table I reveals the similarity not only of the chemical shifts σ_{helix} and σ_{coil} for the α, β, and γ protons in the helical and coil state, respectively, but also of $\Delta\sigma_{h\rightarrow c} = \sigma_{coil} - \sigma_{helix}$, the change in the chemical shift on undergoing a helix–coil transition when the concentration of trifluoroacetic acid is increased.

B. Poly(β-benzyl-L-aspartate). The chemical shifts for $(BzAsp)_n$, which forms a left-handed α helix (Blout and Karlson, 1958), are identical with those reported by Bovey (1968) in solutions of trifluoroacetic acid and chloroform [see Figure 11 of Bovey, 1968].

TABLE I: Changes in the Chemical Shift Arising from the Helix–Coil Transition. $\Delta\sigma_{h\rightarrow c} = (\sigma_{coil} - \sigma_{helix})$.

Resonating Proton	$\Delta\sigma_{h\rightarrow c}$ (in Hz at 100 MHz)			
	$(MeGlu)_n$	$(BzGlu)_n$	$(BzAsp)_n$	Phe_n
Phenyl H		6.2 ± 0.6	2.0 ± 1.0	9.1 ± 1.0
				9.0 ± 1.0
Benzyl CH_2 or CH_3	−0.8 ± 0.6	4.1 ± 0.7	1.3 ± 1.0	
α-CH	57.1 ± 2.0	59.6 ± 2.0	22.6 ± 1.6	46.8 ± 1.7
γ-CH_2	−1.3 ± 1.6	−0.9 ± 1.5		
β-CH_2	−17.4 ± 1.6	−17.8 ± 1.5	3.2 ± 1.4	−7.4 ± 1.5

C. Poly(L-phenylalanine). The chemical shifts of the various protons of Phe_n [which is believed to form a right-handed α helix (Peggion *et al.*, 1969)] as a function of solvent composition at 28° are given in Figure 3, and are in agreement with previous work (Conti and Liquori, 1969) on this polymer. An unusual feature of the nuclear magnetic resonance spectra of Phe_n is that the resonances of the phenyl protons are split into two peaks with a relative integrated intensity of approximately 3:2. The structure of these peaks, shown in Figure 4, is essentially the same as that of the phenyl protons in polystyrene (Bovey *et al.*, 1965). However, there are several splittings in both peaks observed for polystyrene which are not resolved in the spectrum of Phe_n. The spectra of the phenyl protons, shown in Figure 4, can be represented as AA′BB′C spin systems (Bovey *et al.*, 1965), in which the ortho (AA′), meta (BB′), and para (C) protons are affected to differing degrees by adjacent rings. The ortho protons, appearing as the upfield peak, are shifted by the ring current effect to a much greater extent than the meta or para protons. This splitting is maintained in the random coil form, with $\Delta\sigma_{h\rightarrow c}$ for the upfield peak being identical with $\Delta\sigma_{h\rightarrow c}$ for the downfield peak.

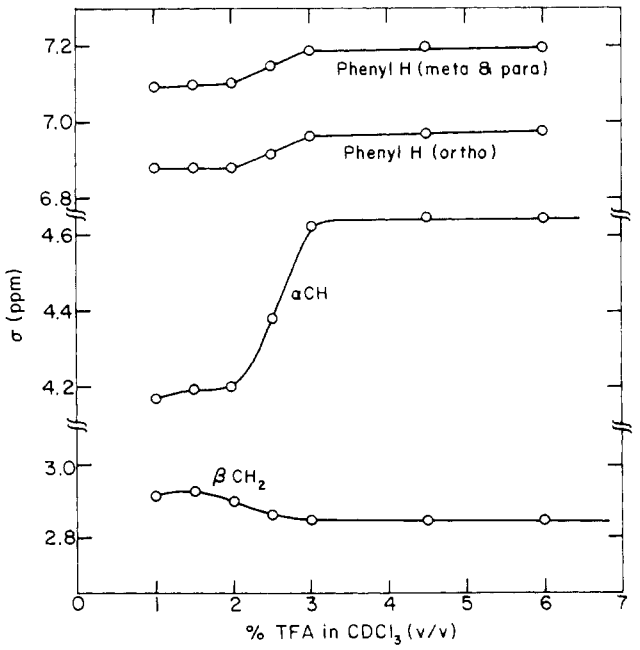

FIGURE 3: Chemical shifts of poly(L-phenylalanine) (Phe_n), at 0.07–0.1 M, in solutions of different concentrations of trifluoroacetic acid and $CDCl_3$, at 28°. Values of the chemical shift (σ) are given in ppm from the internal reference Me_4Si.

Discussion

The general features of nuclear magnetic resonance spectra of polyamino acids in helical and random coil conformations have been described for a variety of different polymers (Markley *et al.*, 1967; Bovey, 1969). The major difficulty in measuring chemical shifts in these spectra is that rather broad lines (10–20 Hz) are exhibited not only by the backbone proton resonances but also by side-chain resonances as well. This broadening arises, in part, from the low mobility of side-chain atoms, especially in β or γ positions (Joubert *et al.*, 1970), and in the backbone of helical conformations.

In those cases where line widths are sufficiently narrow to allow precise measurement of line positions, it is of interest to follow changes of chemical shifts through the helix–coil transition. In most cases, side chains can be expected to be randomly oriented in those polymers whose backbone is randomly coiled. However, in those polymers in which the backbone assumes a helical conformation, the side chains either can be randomly oriented or can take on a particular, low-energy conformation, perhaps best thought of as a transitory species, *i.e.*, the side-chain dihedral angles, which might otherwise be randomly distributed in conformation space, are biased in a statistically favored conformation. Since chemical shifts of side-chain protons represent average over all conformations, they reflect this bias in environment. It is this statistically favored conformation about which we gain information from the nuclear magnetic resonance measurements reported here.

When trifluoroacetic acid induces a helix–coil transition, the change in the chemical shift of side-chain protons, $\Delta\sigma_{h\rightarrow c} = \sigma_{coil} - \sigma_{helix}$, may be expressed as a sum of the following three terms: (1) $\Delta\sigma_{solv}$, the change in the chemical shift arising from solvation of the backbone and/or side chain, and, in addition, from the exposure of sterically blocked protons to the solvent; (2) $\Delta\sigma_{back}$, the change in the chemical shift arising from the disruption of the backbone-side-chain interactions of the helical conformation; and (3) $\Delta\sigma_{side}$, the change caused by the disruption of the side-chain–side-chain interactions of the statistically favored conformation when the side chains are attached to helical backbones. In the convention used here, each of these three contributions to $\Delta\sigma_{h\rightarrow c}$ is positive for a downfield change in the chemical shift upon undergoing a transition from the helical to the random coil form. The main purpose of this paper is to obtain $\Delta\sigma_{side}$ from the observed $\Delta\sigma_{h\rightarrow c}$, and thereby obtain information about the conformation of the side chains of helical polymers.

Many of the conclusions concerning side-chain structure made in the following discussion are based on the nuclear magnetic resonance studies of various N^5-(ω-hydroxyalkyl)-L-glutamines by Joubert *et al.* (1970), in which the helix–coil

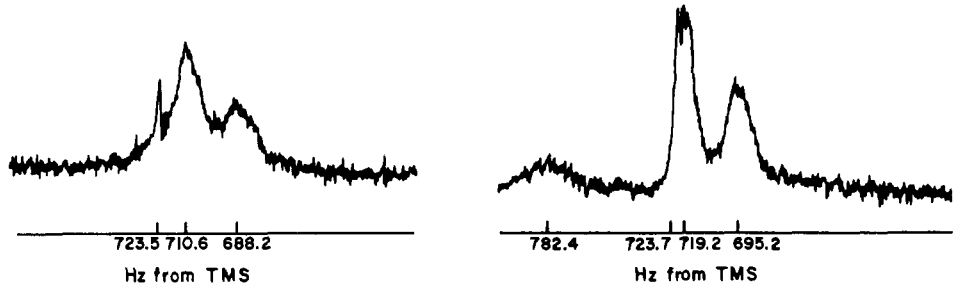

FIGURE 4: The nuclear magnetic resonance peaks of the phenyl protons in poly(L-phenylalanine) in the helical conformation at 2% trifluoroacetic acid (v/v) in $CDCl_3$ (left) and in the random coil conformation at 3% trifluoroacetic acid (v/v) in $CDCl_3$ (right). The sharp peak (at ~723 Hz) in both spectra is residual $CHCl_3$ in the deuterated solvent, and the broad peak at 782 Hz in the coil form is the NH resonance. (0.09 M polymer concentration; 28°).

transition was induced by a temperature change only. It was concluded that, for these polymers, the chemical shifts of side-chain protons are temperature independent and, furthermore, are independent of the helix content of the backbone. That is, all three contributions to $\Delta\sigma_{h\rightarrow c}$ ($\Delta\sigma_{solv}$, $\Delta\sigma_{back}$, $\Delta\sigma_{side}$) were found to be negligible, and the side-chain chemical shifts remained constant throughout their experiment. All of these contributions can not be neglected in the work reported here, since we expect to see the effect of the trifluoroacetic acid in $\Delta\sigma_{solv}$ and the ring current effect of the aromatic groups in $\Delta\sigma_{side}$. For each polymer, however, the contribution of $\Delta\sigma_{back}$ is neglected, in analogy with the work on the glutamines. If $\Delta\sigma_{back}$ were important, it would certainly be evident at the β carbon, but the chemical shifts of Joubert *et al.* (1970) show no such effect at any side-chain position. Consequently, the important contributions to $\Delta\sigma_{h\rightarrow c}$ are taken to be $\Delta\sigma_{solv}$ and $\Delta\sigma_{side}$.

With the above general remarks, we may now discuss the results for $(BzGlu)_n$, poly(γ-methyl-L-glutamate) $(MeGlu)_n$, $(BzAsp)_n$, and Phe_n.

A. Poly(γ-benzyl-L-glutamate). 1. EVIDENCE FOR EXTENDED SIDE CHAINS. As previously discussed (Joubert *et al.*, 1970), the large down-field shift in the α-CH resonance when $(BzGlu)_n$ and $(MeGlu)_n$ are converted into the random coil ($\Delta\sigma_{h\rightarrow c}$ being the same in both polymers) may be attributed to modification of the environment of the α-CH protons of *nonhelical* residues due to changes in both the helix content of the polymer and to thermally induced changes in the conformation of the random coil. But these two polymers behave similarly not only with respect to their α-CH resonances, but also with respect to the resonances of their β-CH_2 and γ-CH_2 protons (see Table I for similarity of $\Delta\sigma_{h\rightarrow c}$ values). This implies that, whatever the preferred conformation of the side chains in the α helix, it is possible to eliminate certain structures in which the phenyl ring is adjacent to the α, β, or γ protons. If such conformations were present, the values of $\Delta\sigma_{h\rightarrow c}$ for $(BzGlu)_n$ would differ from those of poly(γ-methyl-L-glutamate) $MeGlu_n$ because of ring current effects, which are not observed. Therefore, while the side chains may have a preferred orientation in helical $(BzGlu)_n$ in $CDCl_3$–trifluoroacetic acid solutions, as implied in the nuclear magnetic resonance observations of Bradbury and Stubbs (1968), it is not the transverse or longitudinal side-chain conformations found by conformational energy calculations for the polymer *in vacuo* (Yan *et al.*, 1968).

This study of $(BzGlu)_n$ differs from the work of Joubert *et al.* (1970) in the following two aspects. The helix–coil transition was induced by the addition of strong acid and, instead of glutamine derivatives, the benzyl ester of glutamic acid was used. Consequently, for $(BzGlu)_n$ we must account for $\Delta\sigma_{solv}$ from the $CDCl_3$–trifluoroacetic acid solvent and for $\Delta\sigma_{side}$ of the *benzyl group only*. The amide group in the side chains of the poly(hydroxyalkylglutamine) derivatives was found to give no contribution to the value of $\Delta\sigma_{h\rightarrow c}$. Similarly, we assume that the side-chain ester groups in $(BzGlu)_n$ and $(MeGlu)_n$ have little effect on the values of $\Delta\sigma_{h\rightarrow c}$ because the ester has a lower dipole moment than the amide group (Ooi *et al.*, 1967) and therefore exerts a smaller electric field effect on the chemical shift of nearby protons. Furthermore, since $\Delta\sigma_{h\rightarrow c}$ is very small for the γ-CH_2 and benzyl-CH_2 protons of $(BzGlu)_n$, and is nearly zero for the γ-CH_2 and CH_3 protons of $(MeGlu)_n$, there does not appear to be any change of solvation of the ester group at the helix–coil transition. From this same evidence, and from the lack of any noticeable ring current effect in $(BzGlu)_n$, it is unlikely that there is a large change in the degree of exposure of the benzyl group to the solvent at the helix–coil transition. We conclude that, whatever the conformation of the side chain is, it seems to be extended into solution in the helical polymer. As mentioned above, we need to account for $\Delta\sigma_{side}$ due to the phenyl ring only, and for $\Delta\sigma_{solv}$. Since the side chain of $(BzGlu)_n$ seems to be extended, we see no evidence of interaction between the phenyl ring and the β-CH_2 protons. Consequently, we set $\Delta\sigma_{side} = 0$ for the β-CH_2 protons and attribute the entire shift of -0.178 ppm of these protons to $\Delta\sigma_{solv}$. This value of $\Delta\sigma_{solv} = -0.178$ ppm for the β protons will be used in the analysis of the Phe_n spectrum. There is no evidence of a solvent effect for the other side-chain protons.

An extended side-chain conformation in $CDCl_3$–trifluoroacetic acid solvent is not unexpected since organic solvents are able to compete with the backbone for interactions with the nonpolar side chains. In other words, in the absence of hydrophobic interactions, there is no major interaction favoring a compact structure. Indeed, a structure with extended side chains could be favored entropically as well as energetically in this organic solvent. This is pointed out by Yan *et al.* (1968) with the observation that the more compact, longitudinal side-chain conformations computed for $(BzGlu)_n$ and $(MeGlu)_n$ *in vacuo* are more likely to be favored in an aqueous environment than in an organic medium. Calculations for polypeptides *in vacuo* exhibit energy minima in which side chains interact favorably with the backbone. Similar side-chain–backbone interactions are also expected for polypeptides in aqueous solution in which nonpolar side chains minimize their contact with water by forming hydrophobic bonds.

2. EVIDENCE FOR RELATIVE SIDE-CHAIN ORIENTATION. Accepting that the side chains are extended, we wish to comment on the relative positions of the side chains with respect to each

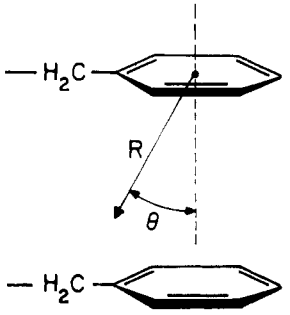

FIGURE 5: Relative orientations of two side-chain groups in α-helical poly(γ-benzyl-L-glutamate) $(BzGlu)_n$ consistent with the chemical shift data. The planes of both phenyl rings are parallel. The dotted line represents the axis of symmetry assumed for the anisotropic magnetic susceptibility of the phenyl ring. Values of θ near 0° and 180° are favored. As implied by the direction of the vector R, the ring current of the phenyl group can affect the resonance of the CH_2 protons.

other. The experimental evidence which is used for this purpose is the value of $\Delta\sigma_{h\rightarrow c}$ for the phenyl protons in $(BzGlu)_n$, and to a lesser extent the benzyl CH_2 protons, shown in Figure 1. Since we have determined that the side chains are extended into solution in the helical polymer and that the side chains do not appear to be exposed suddenly to the solvent at the helix–coil transition, it is difficult to account for this value of $\Delta\sigma_{h\rightarrow c}$ in terms of solvent effects. Instead, it is possible to show that a shift of this magnitude could be caused by a neighbor anisotropy effect arising either from intramolecular interactions or from intermolecular interactions accompanying aggregation. If the change in the chemical shift is attributable to such an anisotropy effect, then the term $\Delta\sigma_{side}$ arising from a disruption of side-chain structure in the helix can be written (Pople *et al.*, 1959) as

$$\Delta\sigma_{side} = \frac{\chi_{||} - \chi_{\perp}}{3\langle R^3\rangle}\langle 1-3\cos^2\theta\rangle \qquad (1)$$

where $\chi_{||} - \chi_{\perp}$ is the anisotropy of the magnetic susceptibility of an axially symmetric neighboring group. R is the distance of the resonating proton from this group, and θ is the angular displacement from the symmetry axis (see Figure 5). The symbol $\langle\cdots\rangle$ indicates averages of the quantities inside over the motion of the side chains. Since $\chi_{||} - \chi_{\perp}$ is negative for the phenyl ring (Hoarau *et al.*, 1956), the sign of $\Delta\sigma_{side}$ will be positive for negative values of $\langle 1-3\cos^2\theta\rangle$. A positive value of $\Delta\sigma_{side}$, corresponding to a downfield change in σ upon disruption of the side-chain structure, occurs when θ is more often near 0° and 180° than near 90° and 270°. Such values of $\Delta\sigma_{side}$ are observed for the benzyl-CH_2 and phenyl protons of $(BzGlu)_n$ (see Figure 1 and Table I). For $(MeGlu)_n$, where this effect is absent because there is no phenyl ring, the value of $\Delta\sigma_{side}$ is essentially zero for the CH_3 protons. Thus we conclude that the side chains of helical $(BzGlu)_n$ in $CDCl_3$–trifluoroacetic acid solution are extended, having a relative orientation of the phenyl rings as shown in Figure 5, with θ having a statistical preference to be near 0°.

It is of interest to compare the side-chain conformations of $(BzGlu)_n$, $(MeGlu)_n$, and $(BzAsp)_n$, as far as their contributions to the stability of these helices and to $\Delta\sigma_{h\rightarrow c}$ are concerned. Using as a criterion of stability the concentration of trifluoroacetic acid required to disrupt the helical form, the helix–coil transition at 50° occurs at 15–20% and at 10–15% trifluoroacetic acid (in $CDCl_3$) for $(BzGlu)_n$ and $(MeGlu)_n$, respectively. This greater stability of $(BzGlu)_n$ over $(MeGlu)_n$ can be attributed to a difference in side-chain interactions. Undoubtedly, one of the contributions to the greater stability of $(BzGlu)_n$ is provided by more efficient shielding (by the bulkier benzyl group) of the hydrogen-bonded helical backbone from the solvent. Another contribution would be the nonbonded interactions between the phenyl rings in the conformation postulated in Figure 5. On the other hand, the bulky side chains of $(BzAsp)_n$, which might also shield the backbone, do not provide as much stability as observed for $(BzGlu)_n$, since $(BzAsp)_n$ undergoes the helix–coil transition before 5% trifluoroacetic acid (in $CDCl_3$), as shown in recent studies (Bovey, 1968). Presumably, the nonbonded phenyl–phenyl interactions, which are present in $(BzGlu)_n$ and contribute to its stability, are absent in $(BzAsp)_n$ (and, of course, absent in $(MeGlu)_n$ which has no phenyl group). The absence of a phenyl–phenyl interaction in $(BzAsp)_n$ and $(MeGlu)_n$ is consistent with the observation in Figure 2 and Bovey's (1968) work that $\Delta\sigma_{h\rightarrow c} \cong 0$ for the phenyl and CH_3 protons of $(BzAsp)_n$ and $(MeGlu)_n$, respectively. Thus, unlike $(BzAsp)_n$ and $(MeGlu)_n$, there is evidence to indicate that $(BzGlu)_n$ exhibits a statistical helical arrangement of the phenyl rings around the inner backbone helix, because of the relative orientations of the side chains as shown in Figure 5.

Goodman *et al.* (1963) and Bradley *et al.* (1966) have provided evidence for a helical arrangement of side chains from the Cotton effect at 370 mμ of copolymers of β-benzyl-L-aspartate and β-(*p*-nitrobenzyl)-L-aspartate, but found none in $(BzAsp)_n$ or in nitrated $(BzGlu)_n$. Further evidence for a statistical helical arrangement of the side chains of $(BzGlu)_n$ was obtained by Parry and Elliott (1967) from the X-ray diffraction pattern of oriented solutions of this polymer in dimethylformamide.

Finally, while $(BzGlu)_n$ has a tendency to aggregate, which has been observed by a number of techniques, including work on a cholesteric liquid crystal phase (Parry and Elliott, 1965), the behavior of $\Delta\sigma_{h\rightarrow c}$ for the benzyl-CH_2 and phenyl peaks of $(BzGlu)_n$ cannot be attributed to aggregation. First of all, the chemical shifts in the helical polymer are independent of $(BzGlu)_n$ concentration in the range of 1–5% (w/v). Secondly, whereas increasing amounts of trifluoroacetic acid would be expected to alter any possible degree of aggregation, these chemical shifts do not change as the concentration of trifluoroacetic acid is increased up to the range of the helix–coil transition (Figure 1).

B. Poly(β-benzyl-L-aspartate.) We have already noted that $\Delta\sigma_{h\rightarrow c}$ is nearly zero, within experimental error, for the phenyl and benzyl-CH_2 protons of $(BzAsp)_n$, and that there is probably no phenyl–phenyl interaction in this polymer, analogous to that postulated in Figure 5 for $(BzGlu)_n$. Further discussion of the $\Delta\sigma_{h\rightarrow c}$ values for $(BzAsp)_n$ in Table I is reserved for a later publication in which these values will be compared to those of the corresponding methyl ester.

C. Poly(L-phenylalanine). 1. EVIDENCE FOR RELATIVE SIDE-CHAIN ORIENTATION. The existence of the ring current effect, implied by the splitting of the phenyl proton peak of Figure 4, indicates that the rotation of the phenylalanine side chain is restricted; that is, certain side-chain conformations are statistically favored. This splitting appears for both the helical and random coil forms indicating the existence of statistically favored side-chain conformations even in the random coil. This result supports the suggestion of Auer and Doty (1966) that the restricted rotation of the phenylalanine side chain is largely due to unfavorable side-chain–backbone interactions

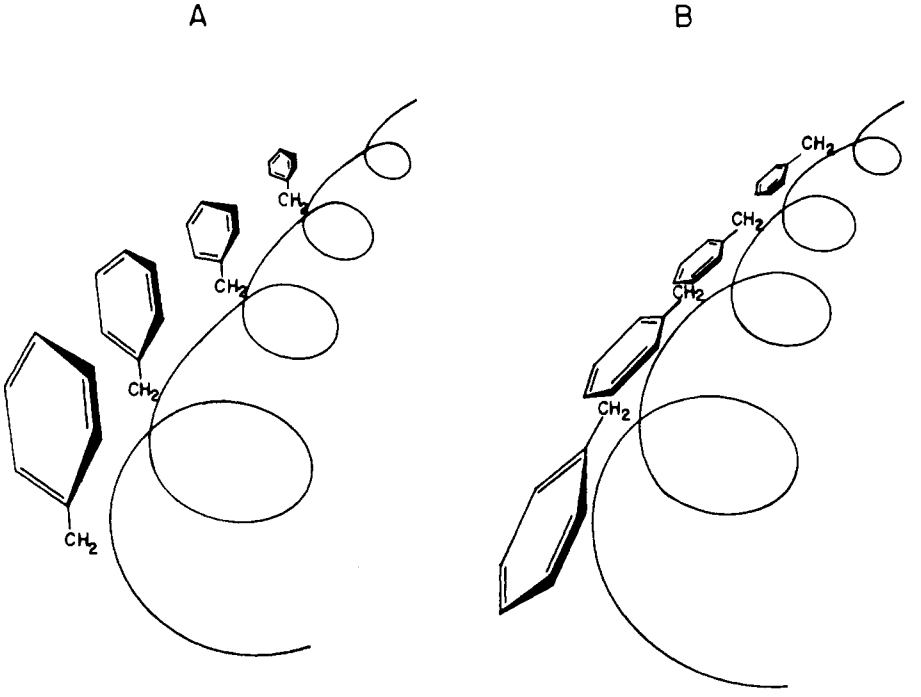

FIGURE 6: (A) Simplified diagram of a proposed structure of poly(L-phenylalanine) consistent with chemical shift data. Overlapping of eclipsed phenyl rings produces predominantly upfield shifts. The backbone is a right-handed α helix with $\chi_1 = 180°$, $\chi_2 = 90°$. (B) A low-energy structure proposed by Yan *et al.* (1968) for poly(L-phenylalanine) in the environment of a vacuum. Side-by-side or in-line arrangement of phenyl rings produces predominantly downfield shifts for phenyl protons. The backbone is a right-handed α helix with $\chi_1 = 302.6°$, $\chi_2 = 155.3°$. The convention for χ_1 and χ_2 is given by Edsall *et al.* (1966a–c).

rather than to unfavorable interactions between adjacent side chains.

The data of Table I enable us to obtain information about the conformation of the side chains in Phe_n by evaluating $\Delta\sigma_{side}$ for all of the side-chain protons. We make the assumption that certain of the results found for the poly(hydroxyalkylglutamine) derivatives and for $(BzGlu)_n$ are valid for Phe_n. That is, we assume that $\Delta\sigma_{back}$ is negligible for all side-chain protons. In addition, of all the side-chain protons in Phe_n, we assume that only the β-CH_2 protons are affected by $\Delta\sigma_{solv}$. This is consistent with the result on $(BzGlu)_n$ in which, considering the side chains, $\Delta\sigma_{solv}$ is found to contribute only to the β-CH_2 protons. We can then write

$$\Delta\sigma_{side} = \Delta\sigma_{h\rightarrow c} - \Delta\sigma_{solv} - \Delta\sigma_{back} \qquad (2)$$

for each side-chain chemical shift. Using $\Delta\sigma_{solv} = 0.178$ ppm for the β-CH_2 protons, obtained from $(BzGlu)_n$, and the data in Table I for $\Delta\sigma_{h\rightarrow c}$, we obtain the data shown in the last column of Table II. These values of $\Delta\sigma_{side}$ provide a criterion from which to deduce the orientation of the side chains of Phe_n.

It is clear that the ring current (anisotropy) effect makes the major contribution to $\Delta\sigma_{side}$ since there is neither a large electric field effect associated with the side chain of the phenylalanine residue nor is there any other group in the side chain with an anisotropic magnetic susceptibility as large as that of the phenyl ring. Values of $\Delta\sigma_{side}$, calculated from the ring current effect alone, using eq 1, are shown in Table II for two possible conformations of the side chains of Phe_n. These two conformations (shown schematically in Figure 6) are (A) a conformation selected to have low energy (see conformation 3 in Table III) and to reproduce the correct sign of $\Delta\sigma_{side}$, and (B) a low-energy structure proposed by Yan *et al.* (1968) on the basis of conformational energy calculations performed for a polymer *in vacuo*. For these computations, the polymers were considered to have only the given conformation (to avoid having to take an average over many values of R and θ); R was measured from the center of the phenyl ring, and θ was computed from the given values of the side-chain dihedral angles χ_1 and χ_2. The value of $\chi_{||} - \chi_{\perp}$ was taken as that for benzene, *viz.*, -59.7×10^{-6} cm^3/mole (Hoarau *et al.*, 1956).

2. CONSIDERATION OF CONFORMATIONAL ENERGY CALCULATIONS. Actually, there are many conformations (all of which fall in the shaded area of Figure 7) which give positive values of $\Delta\sigma_{side}$ for all side-chain protons, in agreement with the experimental data in Table II; some of these are given in Table III, together with that of Yan *et al.* (1968). The positive values of $\Delta\sigma_{side}$ arise because, as illustrated in Figure 6A, the phenyl

TABLE II: Values of $\Delta\sigma_{side}$ for Poly(L-phenylalanine).[a]

Resonating Protons	$\Delta\sigma_{side}$ (in ppm) Calcd for Conformation A[b]	Calcd for Conformation B[c]	Derived from Experimental Data
Phenyl ortho	0.202	−0.091	0.091 ± 0.010
Phenyl meta and para	0.036	−0.100	0.090 ± 0.010
β-CH_2	0.161	+0.473	0.104 ± 0.030

[a] All side-chain conformations correspond to a *right-handed* α-helical backbone. [b] Point 3 of Figure 7 ($\chi_1 = 180°$, $\chi_2 = 90°$). [c] From Yan *et al.* (1968) ($\chi_1 = 302.6°$, $\chi_2 = 155.3°$).

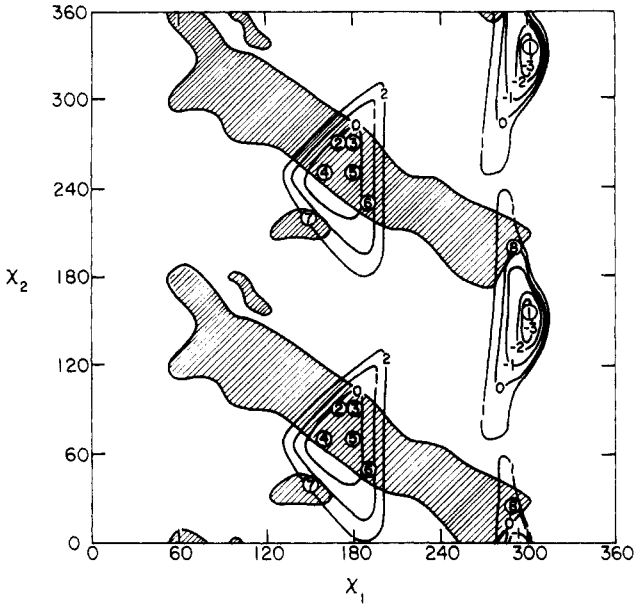

FIGURE 7: Energy contour map for the right-handed α helix (ϕ = 132°, ψ = 123°) of poly(L-phenylalanine) computed from the expression of Gibson and Scheraga (1967a,b) for the potential energy of a polyamino acid in aqueous solution. The energies (on the contour lines) were computed for a polymer containing ten peptide bonds and are given in kcal/mole of residue. The convention is that of Edsall *et al.* (1966a–c). The shaded areas contain those conformations that have nuclear magnetic resonance shifts, $\Delta\sigma_{side}$, which are positive for all side-chain protons (see Table III). The circled numbers represent the eight side-chain conformations listed in Table III.

rings overlap one another in an eclipsed or stacked conformation (in a way suggestive of shingles); *i.e.*, (1–3 $\cos^2\theta$) in eq 1 is negative for such overlapping conformations. On the other hand, the conformation of Yan *et al.* (1968), computed for a vacuum environment, gives negative values of $\Delta\sigma_{side}$ for the phenyl protons because the side-by-side or in-line arrangement of the phenyl rings (see Figure 6) leads to positive values of (1–3 $\cos^2\theta$). For the organic solvent system used here, the conformation must lie in the shaded area of Figure 7.

It is of interest to note that conformational energy calculations can supplement data for $\Delta\sigma_{side}$ to help select the preferred conformation. For this purpose, energy contours are superimposed on the nuclear magnetic resonance data in Figure 7. Presumably, the side chain adopts a conformation which lies both in the shaded region and in a low-energy region. Figure 7, computed for a polymer in water, serves to illustrate the potential combined use of nuclear magnetic resonance and conformational-energy data. It does not apply strictly to the systems studied here, however, since the solvent was $CDCl_3$–trifluoroacetic acid, for which no conformational energy calculations have yet been carried out. Projected experiments on Phe_n in water may indicate whether the shaded regions will overlap better with the low-energy parts of the contour map.

The errors inherent in computing chemical shifts from the magnetic susceptibility of neighboring groups must be pointed out. As used here in the form of eq 1, this method assumes that the induced magnetic moment arising from the electron distribution of the neighboring group is a point dipole. In the case of the phenyl ring, the position of this point dipole is taken to be at the geometric center of the ring. Didry and Guy (1961) have concluded that the point-dipole approximation leads to errors in computed chemical shifts which may be as large as 30% for protons at distances of 3 Å from the location of the point dipole. Fortunately, none of the intramolecular interactions computed in this paper using eq 1 occurred at distances as small as 3 Å, and the use of a point dipole here leads to more useful results since the accuracy of this approximation improves at larger distances. Consequently, we conclude that such chemical shift calculations in favorable cases can be of some importance as a supplement to conformational-energy calculations in deducing which structure, among a number of low-energy possibilities, is preferred in solution.

TABLE III: Computed Values of $\Delta\sigma_{side}$ for Points of Figure 7.

Point	$\Delta\sigma_{side}$ (in ppm)		
	Ortho	Meta and Para	β-CH_2
1[a]	−0.091	−0.100	0.473
2	0.227	0.053	0.263
3	0.202	0.036	0.161
4	0.084	0.021	0.512
5	0.186	0.038	0.299
6	0.080	0.043	0.186
7	0.009	0.088	0.231
8	0.237	0.056	0.495

[a] This is the conformation of Yan *et al.* (1968).

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Calorimetric Investigation of Inhibitor Binding to Rabbit Muscle Aldolase*

Hans J. Hinz,† Daniel D. F. Shiao,‡ and Julian M. Sturtevant§

ABSTRACT: The enthalpies of binding, ΔH_B, of the substrate-analogous inhibitor D-hexitol 1,6-diphosphate to rabbit muscle aldolase have been measured at different temperatures at pH 7.5, employing a flow microcalorimeter. By using four buffer systems with different heats of ionization, ΔH_i, it has been shown that 1.4 ± 0.2 moles of H^+ per mole of enzyme is absorbed when the enzyme is saturated with inhibitor irrespective of the temperature at which the reaction takes place. The temperature variation of ΔH_B corresponds to a temperature independent heat-capacity change, ΔC_p, of −1100 ± 200 cal deg^{-1} (mole of enzyme)$^{-1}$ at saturation. Calorimetric titration data at 25° have been interpreted on the assumption of independent binding sites to yield a value for the intrinsic binding constant, K_B. The temperature variation of K_B calculated using the observed values of ΔH_B, including ΔC_p, is consistent with that deduced from enthalpy titration curves at other temperatures and is very similar to that reported by Lehrer and Barker (Lehrer, G. M., and Barker, R. (1970), *Biochemistry 7*, 1533) for D-arabinitol 1,5-diphosphate. These results emphasize that the explanation offered by Lehrer and Barker *et al.*, for nonlinear van't Hoff or Arrhenius plots, based on an assumed temperature-dependent equilibrium between two forms of the enzyme, can *in general* be replaced by the simpler assumption of a nonvanishing ΔC_p or $\Delta C_p^{\ddagger}$.

In a recent publication, Lehrer and Barker (1970) reported that various binding and kinetic parameters of rabbit muscle aldolase show a strong dependence on temperature. The parameters studied were the Michaelis–Menten constant, K_m, and the maximum velocity, V_{max}, for the splitting of D-fructose 1,6-diphosphate, and the equilibrium constant, K_I, for the dissociation of an inhibitor, D-arabinitol 1,5-diphosphate. Lehrer and Barker proposed an explanation for the observed temperature effects based on a temperature-dependent transition, centered at approximately 30°, of the enzyme between two forms having markedly different binding and catalytic properties. A similar proposal has been made by Massey *et al.* (1966) for the case of D-amino acid oxidase.[1]

Data such as those reported by Lehrer and Barker (1970) can always be rationalized in a very reasonable way on the basis of heat capacity changes accompanying binding processes or the formation of activated complexes, without any assumption of multiple forms of the native enzyme. This is illustrated in Figure 1, in which the data given by Lehrer and Barker for the dissociation constant of the aldolase–arabinitol diphosphate complex are reproduced (within the accuracy of reading their Figure 1). The solid curve in the figure was calculated on the basis of the assumptions that the apparent heat

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† Present address: Research Laboratories, Eastman Kodak Co., Rochester, N. Y. 14650.

‡ The recipient of a travel grant from Heinrich Hertz Gesellschaft, Düsseldorf, Germany.

§ To whom to address correspondence.

[1] In calculating the temperature variation of the *equilibrium* distribution of the enzymes between the two hypothesized forms, both Lehrer and Barker (1970) and Massey *et al.* (1966) used assumed enthalpy values which they termed enthalpies of *activation* and represented by the symbol $\Delta H^{\ddagger}$. It seems unfortunate at this late date to use this terminology, which has for decades been reserved exclusively for the analysis of the variation of reaction rates with temperature, for the standard enthalpy change which controls the temperature variation, as expressed in the van't Hoff equation, of an equilbrium constant.