# NUCLEAR MAGNETIC RESONANCE OF SYNTHETIC POLYPEPTIDES

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## ABSTRACT

High resolution n.m.r. spectroscopy of synthetic polypeptides has already been extensively applied to a study of conformations and conformational transitions, particularly the helix–coil transition. Since every atom of an amino acid residue can, in principle, be made to yield a spectrum, the potential of the method for investigating the finer details of conformation far exceeds that of optical methods.

The main chain proton resonances have been shown to be sensitive not only to the helix content (and helix sense in the case of poly aspartate esters) but also to the polydispersity of the sample, giving a direct indication of the conformational heterogeneity at all points through the transition. Earlier suggestions that multiple $\alpha CH$ and $NH$ peaks were the reflection of a slow step in the conversion of a helical residue into a solvated coil state, have now been shown to be unfounded. The $\alpha CH$ shift difference between the two conformational states has been found not to depend on the nature of the polypeptide, but rather to be a function of the solvent system used; in all cases so far observed however, the helical $\alpha CH$ was upfield of the coil. In a situation for which ORD/CD data were ambiguous (poly L tyrosine) this upfield displacement of the peak was itself taken as indicating a coil to helix transition. The same criterion for the existence of a transition has also been applied in the study of the conformations of racemic DL copolypeptides and the method shown to be particularly appropriate for these polymers. The main chain resonances of poly L alanine have long been the subject of discussion, due both to the presence of unusual additional peaks (that are now seen to be in part an end effect) and to the fact that multiple $\alpha CH$ resonances are not observed through the helix–coil transition. It has been shown that this is a direct consequence of the low cooperativity of transition in this case, and other instances of related behaviour have been detailed.

The ability of n.m.r. to study the conformation of the sidechain in addition to that of the backbone, has been exploited the most fully for poly β benzyl L aspartate. This polymer was chosen since it has been calculated that the left handed helix sense is a consequence of specific interactions that involve a strongly preferred sidechain conformation. Analysis of the vicinal and gem spin-coupling led to the result that no such rigidity exists in the sidechain and therefore that a reappraisal is required of the interactions leading to this unusual helix sense.

In AB copolymers, whether block or random, n.m.r. can be used to follow the behaviour of the A and the B components separately. This has been illustrated with a number of glutamate–aspartate copolymers and led to conclusions regarding both helix sense and also the details of the copolymerization process not readily available by other methods.

It may be stated in conclusion that the diversity of conformational problems that have been tackled by high resolution n.m.r. serves to indicate that the method is not merely a supplement to the well known optical techniques but a powerful tool in its own right.

## INTRODUCTION

Although much simpler than proteins, synthetic polypeptides have served as model compounds for a number of conformational features found in proteins. The main approach has been to study synthetic polypeptides by various techniques and establish the physical and optical parameters which are characteristic of regular and defined conformations of the polypeptide chain. Thus ORD/CD and also infra-red spectroscopy have been very successful in defining spectral properties of the α helical and regular extended β conformations in solution and in films. These techniques, however, provide information on the backbone conformation alone and even these data may be unobtainable in ORD/CD if sidechain chromophores obscure those of the main chain. High resolution n.m.r. spectroscopy has the greatest potential of all of the spectroscopic techniques for conformational studies in solution since many different nuclei in a molecule can be studied separately, especially at high fields, and in particular the sidechain spectrum is normally quite separate from that of the main chain. Moreover, each resonance is characterized by three parameters, the shift, multiplicity and relaxation times, all of which may be conformationally dependent. A second advantage of n.m.r. lies in the fact that the time scale of the technique can result in the spectrum being dependent on molecular motions, in particular conformational transitions, whereas the optical spectroscopic techniques always yield a snapshot picture, with each conformation making its own contribution. No information on the molecular dynamics is normally obtainable. This vast potential of n.m.r. is only at the beginning of its realization in biological studies and it is important to lay the ground work for proteins as soundly as possible by studies of synthetic polypeptides.

This paper makes no attempt to review the field but rather to highlight issues of greater importance that can be studied by n.m.r. (such as the details of the helix–coil transition), and also to discuss polymers having unusual or uncertain conformational properties such as poly tyrosine, poly alanine and poly aspartates. Correlation of n.m.r. data with those for the same system studied by ORD/CD (or by infra-red) is important in this work and the ORD parameter used is $b_0$ [$\sim 0°$ for random coil $\pm \sim 630°$ for a left handed (LH) and right handed (RH) helix].

## THE HELIX–COIL TRANSITION

*Figure 1* shows the temperature induced helix–coil transition of that well-known and fairly well-behaved polypeptide poly γ benzyl L glutamate (PBLG) in chloroform/TFA[1]. The apparent linewidths in the random coil form (of the order of 15 Hz for the main-chain αC*H*) are greater than that of small molecules in this solvent system, but not too great to preclude study. It

was early established[2] that segmental motion of the chain was responsible for these relatively sharp lines. The true linewidth of the αC*H* resonance may be about 5 Hz, the remainder being a consequence of amide and β proton coupling. The linewidths of the helical form in *Figure 1* are seen to be similar

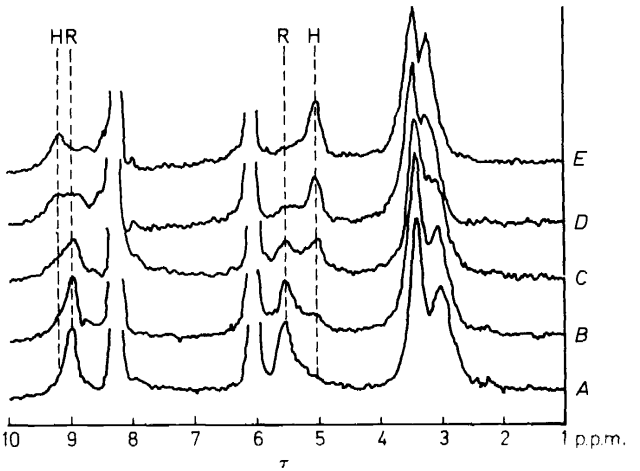



*Figure 1.* Proton magnetic resonance spectra at 100 MHz showing the temperature induced helix–coil transition of poly γ benzyl L glutamate R10, DP = 92, in 8% TFA–92% $CDCl_2$. H—helix, R –random. A, 0°C, $b_0 = 0$; B, 6°C, $b_0 = -100°$; C, 12°C, $b_0 = -220°$; D, 25°C, $b_0 = -340°$; E, 50°C, $b_0 = -470°$. Peak assignments in A: 7.8 p.p.m., amide N*H*; 7.4 p.p.m., aromatics; 5.1 p.p.m., benzyl $CH_2$; 4.6 p.p.m., α*CH*; 2.5 p.p.m., $\gamma CH_2$; 2.1 p.p.m., $\beta CH_2$.

to those of the coil, which is unexpected. This is not a consequence of the increased temperature used to promote the helix form, since decreased TFA concentration at constant temperature has a similar result. The helical form in *Figure 1* cannot therefore be a rigid rod and the relatively sharp lines (in particular the α*CH* proton) must be a result of the presence of a small percentage of coil. Such an interrupted helix would be more compact than a rigid rod, having a more rapid overall correlation time and greater internal flexibility. Furthermore, helix–coil interconversion is thought to be a fast process[3] and the rapid motion of a small coil section along the largely helical molecule could result in linewidths not markedly different from those of the coil. Although this matter will be discussed further, particularly as regards α*CH* linewidths, it may be stated that a full understanding is not yet possible of the motions responsible for the relatively sharp lines of helical polypeptides in TFA-containing solvents. In pure water, however, all polymers so far studied[4, 5, 6] show a distinct broadening of the α*CH* resonance as the helicity rises and it has not been possible to observe the α*CH* peak of for example fully helical poly L glutamic acid or poly L lysine. The greater linewidths observed in water are probably a consequence of intermolecular aggregation that does not take place in most organic solvents. With increasing molecular weight in a defined solvent there is an increase in linewidth of several peaks to a limiting

value and this can be used to estimate molecular weight[7]. Moreover, in both helix and coil, linewidths decrease for all polypeptides as the distance of the proton from the main chain increases, reflecting an increasing molecular flexibility[8]. In pure chloroform linewidths are much greater than in haloacetic acid containing solvents with the amide N*H* and α*CH* resonances often being unobservable (see *Figure 2*). This is a consequence of both aggregation of helices and of an increased rigidity in the helices.

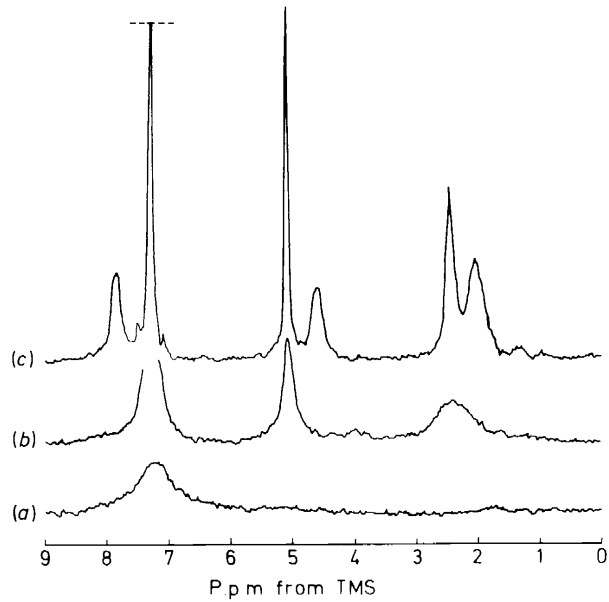



*Figure 2.* Proton magnetic resonance spectra at 100 MHz of PBLG in solution in (a) $CDCl_3$, (b) 2% TFA–98% $CDCl_3$, and (c) 20% TFA–80% $CDCl_3$.

*Figure 1* shows three principal changes in the spectrum as the coil is converted into helix:

(1) the $\beta CH_2$ moves downfield by about 0.3 p.p.m. (see ref. 44, Figure 5),

(2) the α*CH* moves upfield by about 0.5 p.p.m. and

(3) the amide N*H* moves downfield by about 0.2 p.p.m.

This so-called 'double-peak' phenomenon over the helix–coil transition for the main chain amide and α protons[9] has been the subject of much debate and the position will be reviewed here, largely in terms of the α*CH* resonance.

## THE 'DOUBLE-PEAK' CONTROVERSY

For poly γ benzyl L glutamate (PBLG) in chloroform ($CDCl_3$)–trifluoro acetic acid (TFA) the observation of individual helix and coil α*CH* peaks at the mid-transition point separated by about 50 Hz (at 100 MHz) suggests

that the lifetime of a residue in both conformations is in excess of $3 \times 10^{-1}$ sec. However, both theoretical[3a] and experimental studies by dielectric relaxation[10] and temperature-jump[3b] techniques indicate that helix–coil interconversion is at least four orders of magnitude faster than this. Such a rapid rate would result in an averaging of both the αC*H* chemical shift and the peak width over the time a residue spends in the helix and coil states. On the assumption that this averaging is the same over the whole chain, a 'single shifting peak' would thus be observed for the αC*H* resonance as coil is converted into helix. High molecular weight PBLG in $CDCl_3$–TFA does indeed show such a 'single shifting peak' as does poly L alanine (which will be discussed in detail later) but the majority of polypeptides so far studied in various solvent systems including $CDCl_3$–TFA show the 'double peak' αC*H* spectrum. Examples of other polymers studied in $CDCl_3$–TFA are poly β methyl L aspartate (PMLA) and poly L leucine[9], poly β benzyl L aspartate (PBLA)[4, 11, 12], poly L methionine[4, 7, 13, 55] and poly L phenylalanine[14]. 'Double peak' behaviour has also been observed in other solvent systems; poly L arginine in methanol–water[15], poly L tyrosine (PLT)[16] in water–dimethyl sulphoxide (DMSO) and PBLG and PBLA in $CDCl_3$–DMSO[17].

This assignment of the two αC*H* peaks to helix and coil rests on the correlation of the two peak areas with the ORD parameter $b_0$ over the helix–coil transition; this correlation was found to be excellent for PBLG in $CDCl_3$–TFA[1] and is shown in *Figure 3*. A good correlation was also established[13]

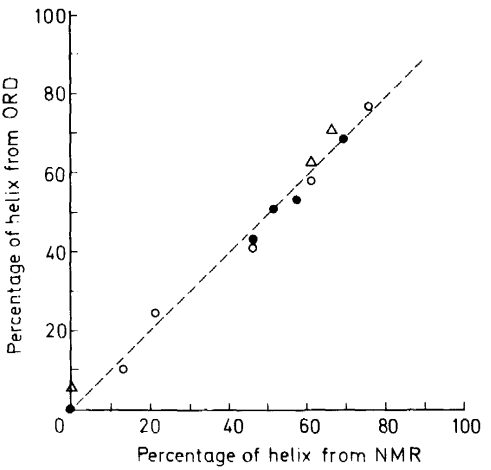



*Figure 3.* Correlation of n.m.r. estimates of helix content (from αC*H* intensities) with ORD estimates (from $b_0$) for three samples of PBLG of DP = 92 (○), DP = 21 (△), and DP = 13 (●).

for poly L methionine at both 60 and 220 MHz using change of TFA concentration rather than temperature. The actual assignment of the two αC*H* components has, however, been challenged by Joubert *et al.*[6] who proposed that the upfield ('helix') peak is due to unsolvated coil residues whilst the

lowfield ('coil') peak is due to solvated coil residues. The shift difference between the peaks is thus attributed entirely to interaction with solvent in the coil state and solvation is postulated to be a slow process since the solvated and unsolvated states are magnetically distinct. The helical residues were postulated to give rise to resonances too broad to be observed. This interpretation has been investigated[18] for PBLG in $CDCl_3$–TFA over the complete helix–coil transition region by measurement of the summed area of both peaks relative to internal standards. The total $\alpha CH$ peak area varied by only eight per cent over the complete transition and it is therefore clear that the $\alpha CH$ resonance of helical residues is almost fully observable under these conditions of measurement.

A second theory of the 'double-peak' phenomenon has been advanced by J. H. Bradbury and co-workers[19] and supported by Tam and Klotz[20]. This theory also involves a slow solvation step as the essential process by which two magnetically distinct $\alpha CH$ proton states are observed. In this case solvation is regarded as complete protonation of the amide group by the acid. The upfield peak is regarded as a composite of unsolvated helix and coil in rapid equilibrium (to accord with the kinetic results) and the low field peak is composed of *protonated* helix and coil residues—also in rapid equilibrium. The shift difference between the two peaks is thus regarded as entirely due to solvation, there being no intrinsic dependence of shift on conformation. Inasmuch as a helix could never maintain a high charge the low-field peak must therefore be due almost entirely to protonated coil. It has been pointed out above that in certain cases an extremely good correlation of peak areas is obtained with the helicity determined from $b_0$ and this must mean that the contribution of unsolvated (unprotonated) coil to the upfield peak must be slight. This scheme therefore reduces to: upfield-peak unprotonated helix, downfield—completely protonated coil. To test this hypothesis, polypeptides have been studied in a non-protonating solvent, dimethylsulphoxide (DMSO) as follows. PBLA is random coil in pure DMSO and on addition of chloroform takes up the usual LH helical conformation[17,27] with a typical 'double peak' being observed near the middle of the transition. PBLG samples of low molecular weight have also shown separate helix and coil peaks in pure DMSO (see *Figure 10*). Since DMSO is a non-protonating solvent it can be concluded that protonation is not required either for promoting the helix–coil transition itself, or for the observation of different and characteristic helix and coil $\alpha CH$ shift values. Infra-red spectroscopy, however, is the most powerful method for establishing whether the PBLG coil in $CDCl_3$–TFA is highly protonated. The amide II vibration is known to be a composite of in-plane NH bending and C—N stretching in the planar *trans* amide group. Protonation on either the nitrogen or oxygen atom would result in disruption of this coupling and complete loss of absorption at $\sim 1550\ cm^{-1}$. *Figure 4* shows the 6 μ region of PBLG in $CDCl_3$ as TFA is added to induce transition to the coil at about 12 per cent acid. At 15 per cent TFA both amide I (at $\sim 1660\ cm^{-1}$) and amide II (at $\sim 1550\ cm^{-1}$) are somewhat broader than in the helix, as expected, but little changed in frequency. There cannot therefore be a large amount of protonation of the amide groups. Solvation of the sidechain ester carboxyl (at $\sim 1735\ cm^{-1}$) by hydrogen bonding is apparent, however.

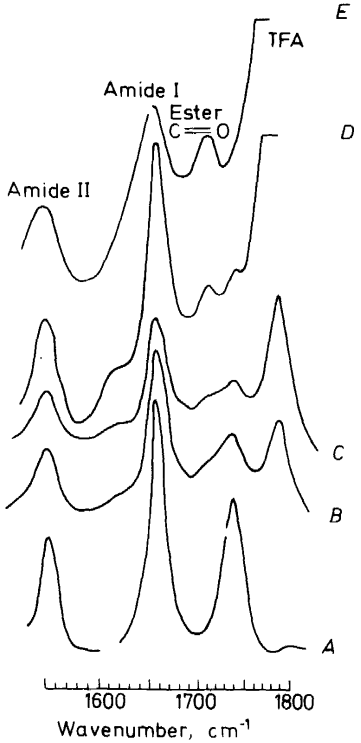



*Figure 4.* Infra-red spectra of 0.2M solutions of poly β benzyl L glutamate in (A) $CDCl_3$; (B) 2.5% TFA–97.5% $CDCl_3$; (C) 5% TFA–95% $CDCl_3$; (D) 10% TFA–90% $CDCl_3$; (E) 15% TFA–85% $CDCl_3$.

A third theory of the 'double peak' phenomenon has been advanced by Ferretti and co-workers[21] who propose helix nucleation as the essential slow step in the kinetic scheme. The very much faster helix and coil propagation rates along the chain are taken by these authors to determine the kinetic times of the order of $\sim 10^{-6}$ sec whilst the longer n.m.r. lifetimes that give rise to separated peaks are calculated to be a consequence of the much slower helix nucleation. The predicted line shape patterns vary with molecular weight and with the assumptions made as to the different nucleation and propagation rate constants. The α*CH* line shapes are characterized by a purely 'random coil' peak that remains fixed in shift and an average helix peak that moves towards the coil peak for all except the lowest molecular weights. Spectra of PBLG of intermediate molecular weights (DPs from 160 to 300) appear to show such an effect[22]. We have studied a very large number of PBLG samples of different molecular weights and find that for samples of DP between 150 and 400 the midpoint of the transition is characterized by a broad resonance having a maximum closer to the coil position for some samples and closer to the helix position for others. With increase of molecular weight the maximum is found to move to a shift midway between the extremes and finally spectra are observed similar to those shown in *Figure 5* for a high

molecular weight sample. The most serious drawback of the theory, however, is that it offers no explanation of how a 'single shifting' peak can be generated and this has been observed both for high molecular weight samples of PBLG and for samples of low polydispersity (see below, *Figures 5* and *9* respectively).

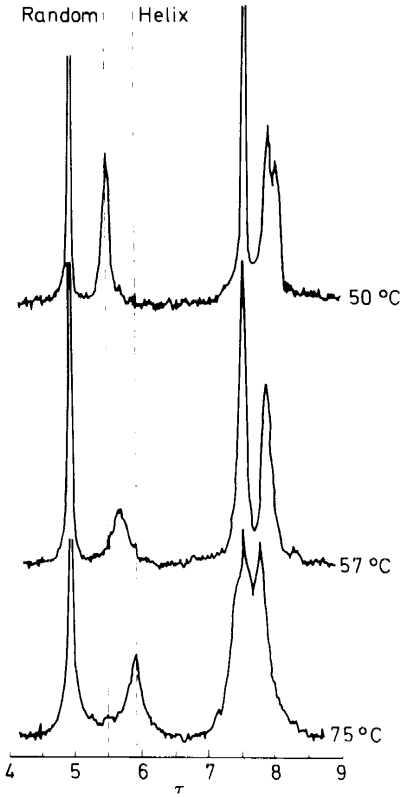



*Figure 5.* 220 MHz proton spectrum of PBLG, DP = 640, in 25% TFA· 75% $CDCl_3$.

Ullman[23] has given a theory of the 'double peak' phenomenon that is the most satisfactory to date since it offers an explanation which is quite independent of polymer or solvent system and predicts in general terms the observed dependence of the α*CH* peak shapes on the molecular weight of the sample. Ullman's theory assumes that there is rapid interconversion of helix and coil states. Two factors could be responsible for the appearance of the 'double peak' according to this approach: first, the free energy difference between helix initiation and helix continuation means that in the helix–coil transition region a residue near the end of a polypeptide chain has a greater probability of being in the coil state than a residue in the middle of the chain, i.e. averaging of helix and coil states is not equivalent for all residues in the chain, resulting in asymmetric peaks. Although this effect would disappear

for very high molecular weight, an averaged and shifting αC*H* peak resulting (as is in fact observed), it is not sufficient to explain the occurrence for low molecular weights of quite separate helix and coil peaks that remain fixed in position through the transition (see *Figure 1*). The second contributing factor (as suggested, independently, by Jardetzky[24, 25]) is molecular weight polydispersity. Under specified solvent conditions the helicity of a given polypeptide chain is strongly dependent on the molecular weight, if that is low. A low molecular weight polydipserse sample therefore in the middle of the helix–coil transition will consist in the main of molecules that are either largely helical (and so contribute to the upfield peak) or largely coil (and so contribute to the lowfield peak). On this basis a good correlation of the upfield 'helix' peak area with $b_0$ (as seen in *Figure 3*) would be observed only for samples having a molecular weight spread broad enough such that under any conditions only a small proportion of the molecules are actually in the process of the helix–coil transition. These postulates have been subjected to an experimental test by the study of several PBLG samples in $CDCl_3$–TFA[26]. The sample of *Figure 6*(a) (R10) having an average DP of 92, was found to have the same $M_w$ and $M_n$, within experimental error, as sample S416 whose spectra are shown in *Figure 6*. A sample of DP ~270

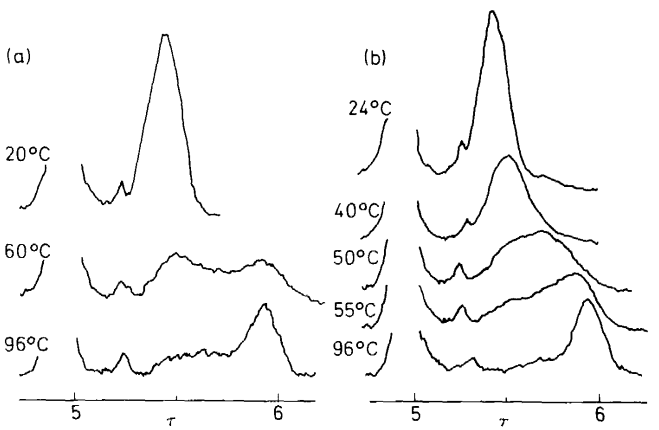



*Figure 6.* 100 MHz proton spectra, αC*H* region, of (a) PBLG, DP = 92 (R10), and (b) PBLG, DP = 100 (S416), both in 20% TFA–80% $CDCl_3$.

(No. 314) gave α C*H* line shapes at 100 MHz intermediate between those of *Figures 5* and *6*(a) and not differing greatly from those of S416 in *Figure 6*(b). Moreover, the αC*H* line shapes of PBLG 314 remained virtually unchanged on remeasurement at 220 MHz. This line shape intermediate between the double peaks of *Figure 1* and the 'single shifting' peak of *Figure 5* therefore represents a continuous range of shift values, i.e. of helicities, within the sample, over the transition region. The high molecular weight sample SP 18.4 (DP ~640) (*Figure 5*) shows what approximates to a 'single shifting peak' in

the transition region, although careful measurement shows that the linewidth rises to a maximum near the middle of the transition. Such changes in linewidth have also been noted for poly L methionine[4]. If polydispersity were the cause of the 'double-peak' phenomenon such that increasing molecular weight results in a change of the line shapes from double peaks to single in the transition region, why then should R10 and S416 give markedly different spectra? Gel permeation chromatography was used to demonstrate that R10 is much more polydisperse than S416 and R10 is therefore very polydisperse in helicity in the transition region. Fractionation of R10 by means of precipitation chromatography has yielded components having weight averages between 20 and 190. *Figure 7* compares the α*CH* spectra of fractions from R10 having weight averages 170 and 45, which ORD measurements

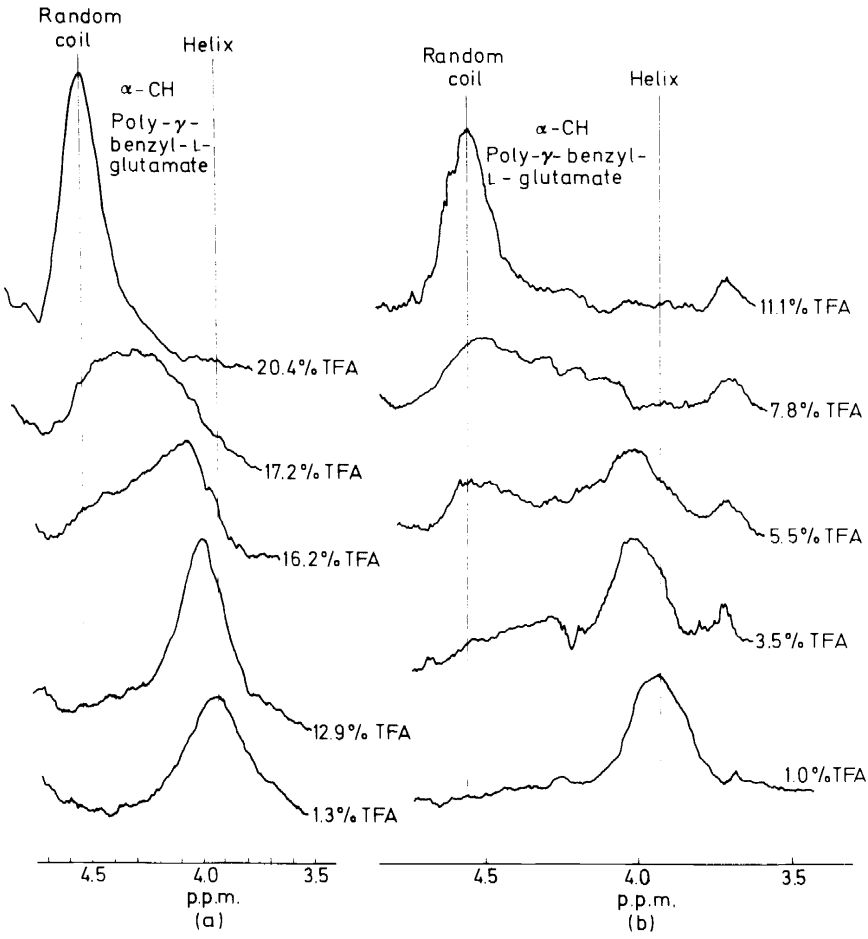



*Figure 7*(a). 100 MHz spectra in TFA–$CDCl_3$ at 22°C of sample (170 mer) fractionated from R10 ($\overline{DP}_w = 92$).

*Figure 7*(b). 100 MHz spectra in TFA–$CDCl_3$ at 22°C of sample (45 mer) fractionated from R10 ($\overline{DP}_w = 92$).

show to have helix–coil transition midpoints at 65 per cent and 40 per cent DCA respectively, i.e. very well separated. That polydispersity is a major cause of the 'double-peak' phenomenon in R10 is thus established. Nevertheless, the 45 mer of *Figure 7* still shows a separation of the helix and coil peaks.

Very recently Nagayama and Wada[27] subjected a sample of PBLG to gel permeation chromatography using DMF as solvent and obtained a fraction having $\overline{DP}_n = 43$. *Figure 8* shows its molecular weight distribution obtained

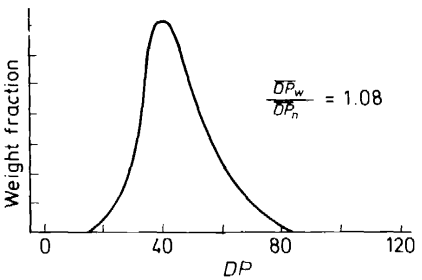



*Figure 8.* Molecular weight distribution of PBLG sample fractionated by GPC in DMF solution.

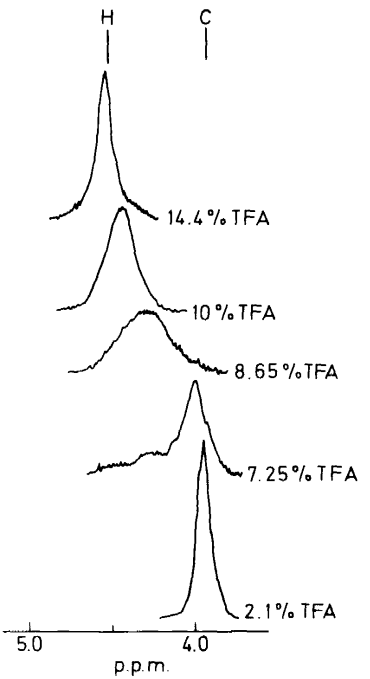



*Figure 9.* 220 MHz αC*H* spectrum of fractionated PBLG sample in the helix–coil transition region in TFA–$CDCl_3$. Polymer concentration, about 3% w/v. H denotes helix position, C, coil position.

by rechromatography. Despite this low molecular weight the appearance of the αC*H* resonance through the helix coil transition ($CDCl_3$–TFA) was essentially that of a single shifting peak (see *Figure 9*). The authors conclude that polydispersity and end effects are the sole cause of the 'double-peak' phenomenon and therefore that no discrepancy whatever exists between the n.m.r. line shapes and kinetic measurements in the helix–coil transition region.

## THE CAUSES OF THE HELIX AND COIL SHIFT-DIFFERENCE

Several solvents have been used to study the helix–coil transition of polypeptides. Data on αC*H* chemical shifts are presented in *Table 1* for those cases in which both fully helical and fully coiled states have been observed. Small errors could, however, be present in certain of the shift values as a consequence of some conformational impurity. Certain data require explanatory comment.

The random coil data on PBLG in $CDCl_3$ and dimethylformamide (DMF) and on PBLA in $CDCl_3$ are indirect, being derived from DL polymers (see also below). The data for PBLG and PBLA in pure DMSO have been obtained in a recent study[17,28] of homo and copolypeptides. *Figure 10* shows the αC*H*

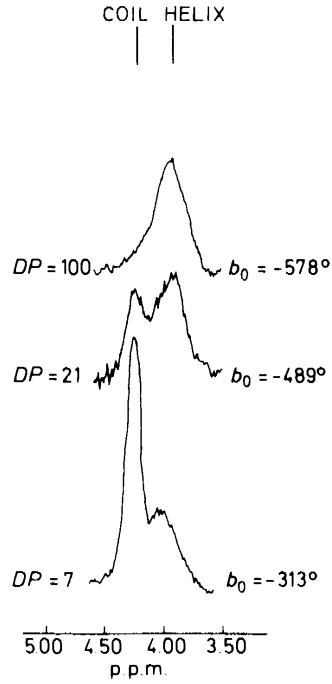



*Figure 10.* 100 MHz spectra, αC*H* region, of three samples of PBLG in $DMSOd_6$ at 30°C.

peak of PBLG samples in DMSO over a considerable molecular weight range and it can be seen that whilst long chains are helical in this solvent, short chains have a large coil component. Pure PBLA is random coil in DMSO[56] and so are random copolymers with PBDG up to 50 per cent

*Table 1*

| Polymer | Solvent at transition midpoint | αCH Helix shift (p.p.m.) | α*C*H Coil shift (p.p.m.) | αCH − $\Delta_{H/C}$ (Helix–coil shift difference) p.p.m. | Ref. |
|---|---|---|---|---|---|
| PBLG | $CDCl_3$–14%TFA | 3.95 | 4.45 | 0.50 | 1, 4 |
| PBLG | Pure $CDCl_3$ | 3.95 | 4.2–4.3 | 0.35–0.25 | 49 |
| PBLG | 27%$D_2O$–TFA | 4.20 | 4.70 | 0.50 | 53 |
| PBLG | 41% formic acid–TFA | 4.25 | 4.70 | 0.45 | 53 |
| PBLA | $CDCl_3$–1.5%TFA | 4.30 (LH) | 4.80 | 0.50 | 11, 43 |
| | | 4.40 (RH) | 4·80 | 0.40 | 11, 43 |
| PBLA | Pure $CDCl_3$ | 4.30 (LH) | 4.3 | 0.0 | 49 |
| Poly D α-amino *n*-butyric acid | $CDCl_3$ 15%TFA | 3.80 | 4.35 | 0.55 | 32 |
| PCLL | $CDCl_3$–9%TFA | 3.90 | 4.45 | 0.55 | 54 |
| Poly L leucine | $CDCl_3$–30%TFA | 4.08 | 4.55 | 0.47 | 32, 36 |
| Poly L methionine | $CDCl_3$–40%TFA | 4.23 | 4.75 | 0.52 | 4, 13 |
| Poly L phenyl alanine | $CDCl_3$–5%TFA | 4.20 | 4.70 | 0.50 | 14 |
| PLT | 10%$D_2O$–DMSO | 4.10 | 4.44 | 0.33 | 16 |
| Poly L arginine | 85%MeOH–$D_2O$ | 4.0 | 4.4 | 0.4 | 15 |
| PBLG | Pure DMSO | 3.95 | 4.26 | 0.31 | 17 |
| PBLA | Pure DMSO | 4.30 (LH) | 4.64 | 0.34 | 17, 28 |
| PBLG | DMF | 4.09 | 4.39 | 0.30 | 11, 49 |
| PGA | $D_2O$/pD 4.8 | 4.2 | 4.3 | 0.1 | 4, 5 |
| PLL | $D_2O$, pD 10.4 | 4.2 | 4.3 | 0.1 | 5 |
| Poly-$N^5$-(4-hydroxy-butyl) L glutamate | $D_2O$ (20°C) | 4.10 | 4.26 | 0.16 | 6 |
| Copoly L glutamic acid[42], L lysine HBr[28], L alanine[30] | $D_2O$(20°C) | 4.2 | 4.3 | 0.1 | 11 |

glutamate. Further addition of PBDG causes the polymer gradually to assume the LH helical form. This establishes the LH helical shift of PBLA as 4.3 p.p.m. (similar to that in $CDCl_3$) and the coil shift as 4.69 p.p.m. The RH helical form of PBLA has not yet been observed in pure DMSO, although in DMSO–60 per cent $CDCl_3$ cosolvent the α*C*H shift is 4.40 p.p.m., as in pure $CDCl_3$.

The data in *Table 1* show that although the αC*H* helix position is always upfield of the coil, the magnitude of this difference $\Delta_{H/C}$ is very dependent on the solvent system, varying from 0.0 to 0.5 p.p.m. The general conclusion to be drawn from *Table 1* is clearly that $\Delta_{H/C}$ values are characteristic of a particular solvent system, rather than of the polymer. This suggests that $\Delta_{H/C}$ largely results from solvation differences between the helix and the coil, rather than being a difference intrinsic between the two conformations.

## WATER SOLUBLE POLYPEPTIDES

*Table 1* shows that in water the dependence of the αC*H* shift on helix content is lower than in organic solvent systems. The peak displacement has been followed for both poly L glutamic-acid (PGA) and poly L lysine (PLL) over the helix–coil transition[4,5], but in both cases aggregation of helical polymer with consequent line broadening prevented observation of the fully helical state. The value of $\Delta_{H/C}$ for these polymers was indicated to be not less than 0.1 p.p.m. A copolymer of molar composition (L-glutamic acid[42], L-lysine HBr[28], L-alanine[30]) has also been studied in water[5], and the helicity varied by both pH and temperature change. Only a small upfield shift of the αC*H* peak was noted on helix formation amounting (on measurement at 220 MHz[11]) to 0.04 p.p.m. as the helicity changed from 32 to 71 per cent. This implies a value of ~0.1 p.p.m. for the complete transition. Joubert *et al.*[6] have studied several glutamine derivatives in water the helicity of which is dependent on temperature. This allowed the helix–coil transition to be studied without any complicating effects due to sidechain ionizations, though aggregation of helical polymer was just as evident as with PGA or PLL. In the case of poly *N*-(hydroxybutyl) L glutamine (PHBG) at 20°C a shift of 4.26 p.p.m. was observed for the coil form and 4.13 p.p.m. for a helix content of 67 per cent. The extrapolated value of $\Delta_{H/C}$ for the complete transition of PHBG was 0.16 p.p.m. In all the above studies in water the αC*H* resonance appeared to move as a 'single shifting peak' and did not exhibit any obvious 'double-peak' character. Since, however, the displacements are of the same order as the linewidths and aggregation broadening takes place on helix formation, 'double peak' behaviour would be largely obscured.

The above results show that for protein conformational studies in water the αC*H* shift is not likely to be of great value as a consequence of its low dependence on helicity.

### Poly L alanine

The spectrum of poly L alanine (PLA) in $CDCl_3$–TFA–DCA has been the subject of much discussion in the literature for two reasons. First, as seen from *Figure 11*, the principal αC*H* resonance has the appearance of a 'single-shifting peak' even for samples of low molecular weight[4,29,30,31]. This behaviour is normally associated only with polymers of the highest molecular weight (e.g. see *Figure 5*). The PLA peptide N*H* resonance remains approximately constant in shift over the same range of solvent composition from 30 to 100 per cent TFA, moving only ~0.08 p.p.m. upfield. This approximate constancy of the N*H* peak is probably the result of a balance between upfield displacement by ~0.2 p.p.m. (the value observed for PBLG in *Figure*

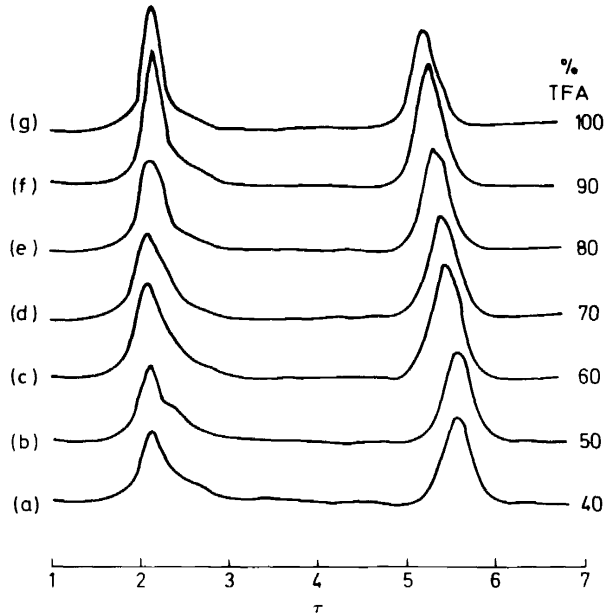



*Figure 11.* 60 MHz spectra of poly L alanine in TFA–$CDCl_3$. Amide N*H* and α*CH* protons.

*1*) due to the breakdown of the helical conformation and a downfield displacement of the peak by about the same amount due to the overall increase of TFA, as discussed above for the α*CH* proton. The small net displacement of the N*H* peak nevertheless shows the same form as the change in $b_0$. Over the same solvent composition the single α*CH* resonance moves ~0.30 p.p.m. downfield. This too correlates with the $b_0$ changes, although the magnitude of the displacement is in this case augmented by the bulk TFA effect. Thus although the PLA transition is not sharp and differs thereby from PBLG, there is no reason on the basis of the above to doubt that the principal α*CH* and N*H* peaks of PLA represent an average of helix and coil states and are therefore a good indication of conformation, in the same way as for high molecular weight PBLG. The reasons underlying the observation of a 'single shifting' α*CH* peak for PLA rather than the more usual 'double-peaks' will be discussed later.

Detailed study of PLA[31, 32] reveals, however, that there is a subsidiary N*H* peak (at ~7.7 p.p.m. in 80 per cent TFA–$CDCl_3$) and a subsidiary α*CH* peak (at ~4.7 p.p.m. in 80 per cent TFA–$CDCl_3$). These peaks are denoted by the letter S in *Figures 12* and *13*. Both these peaks move to lowfield by 0.1–0.2 p.p.m. over the TFA range of 30 to 100 per cent as can be seen from *Figure 12*. Their shifts are just those found for the N*H* and α*CH* peaks of poly DL alanine[30] in $CDCl_3$–TFA and their areas grow slightly at the expense of the main peaks with increasing TFA. For these reasons Ferretti and Paolillo[31] assigned the subsidiary peaks to random coil, and the main peaks to fully helical polymer thereby concluding that PLA is largely helical in TFA and $b_0$

is no guide to the conformation. If, however, a wide range of molecular weights of PLA is studied then it is found that the subsidiary peaks become reduced in intensity the more the molecular weight is increased. This is shown for the N*H* resonance in $CDCl_3$–80 per cent TFA in *Figure 13*. This figure also

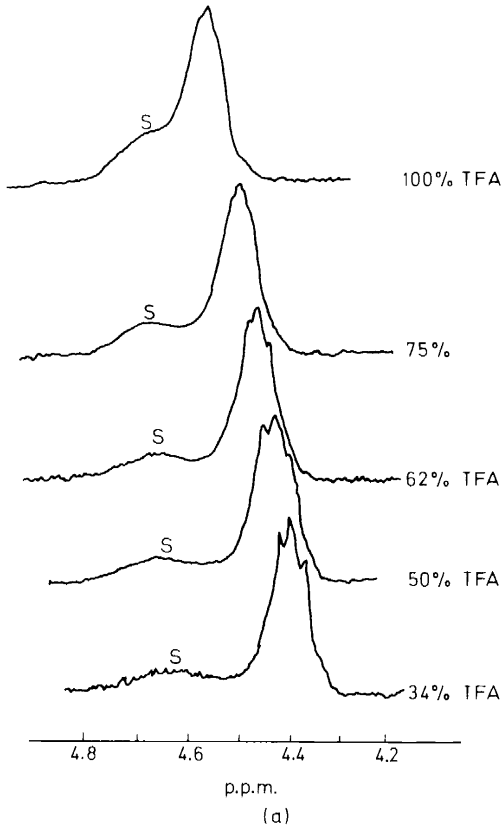



*Figure 12*(a). 220 MHz spectra, α*CH* region, of poly D alanine, $\eta_{sp}/c$ = 9.04 dl $mol^{-1}$, in TFA–$CDCl_3$.

shows the coincidence of the subsidiary peak shift with that of poly DL alanine. This dependence on molecular weight and the correspondence of the shift with that of poly DL alanine suggests that the subsidiary peaks can be assigned to PLA in some coil structure taken up by low molecular weights and/or ends of chains.

If these interpretations of the PLA peaks are correct it implies a coexistence of two states: (i) virtually pure 'random coil', and (ii) rapidly interconverting helix and 'coil', each of which gives rise to its own distinct peak. The reasons underlying this apparent separation of states may be just those given above

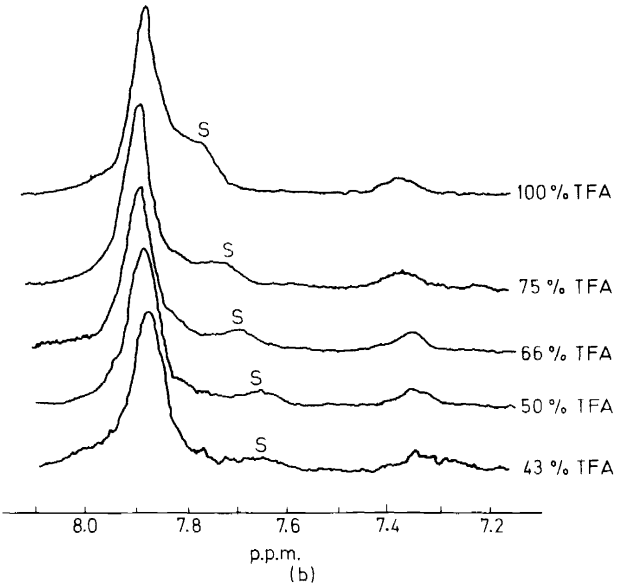



*Figure 12*(b). 220 MHz spectra, N*H* region, of poly D alanine, $\eta_{sp}/c = 9.04$ dl mol$^{-1}$, in TFA–$CDCl_3$.

for the 'double-peak' α*CH* resonance of PBLG in that there can be two contributions. The first factor is polydispersity, as a result of which the two states exist in separate molecules (low molecular weights favouring the highly 'coiled' state, and higher molecular weights the helix–coil interconversion state). In the absence of any fractionation of PLA we cannot yet assess this contribution. The second factor is end effects in that residues near the ends of the chains have an enhanced probability of being in the purely 'coiled' state. We have investigated[33] this factor by studying B—A—B copolymers, where A represents poly L alanine and B represents PBLG, PBLA or poly ε-carbobenzoxy L lysine (PCLL), in $CDCl_3$ from 0 to 100 per cent TFA. The important results of this study were:

(1) that the α*CH* peak moved downfield as a single shifting peak from ~4.1 p.p.m. in $CDCl_3$ to ~4.6 p.p.m. in 100 per cent TFA. These are shift values close to those observed for the helix–coil transition of PBLG in $CDCl_3$–TFA. *Figure 14* shows expanded spectra for a PBLG block copolymer with PLA which illustrate this point.

(2) The α*CH* shift paralleled closely the changes in $b_0$. *Figure 15* shows a decomposition of $b_0$ data for a PBLG block copolymer with PLA and the corresponding α*CH* shift data.

(3) Despite the fact that the PLA block was of low molecular weight there was no sign of the 'subsidiary' α*CH* or N*H* peaks. This is most clearly demonstrated for the α*CH* peak in spectra of PBLA block copolymers since the PBLA α*CH* is well removed from that of PLA. Since the PLA in these

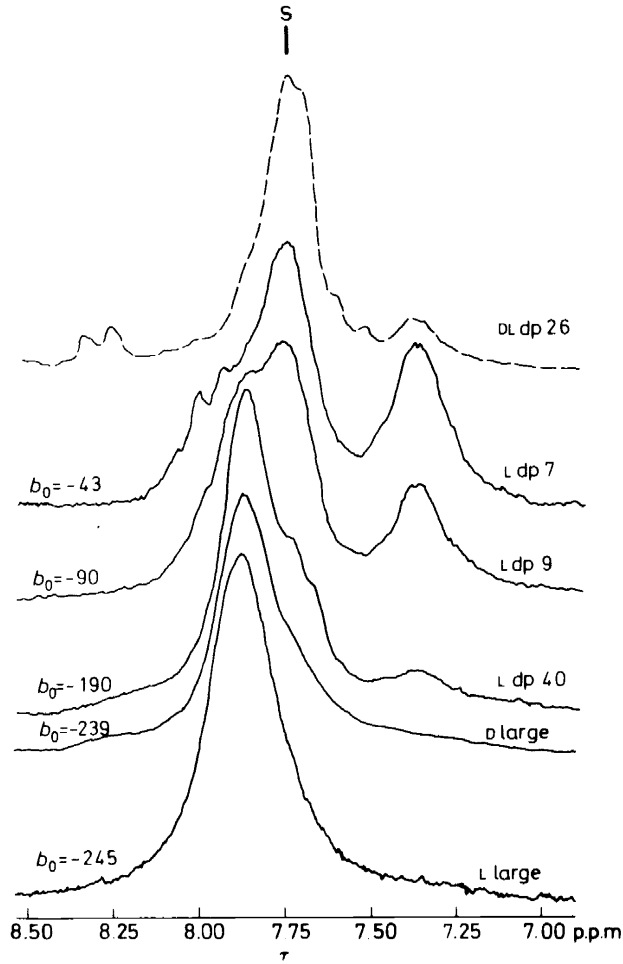



*Figure 13.* 100 MHz N*H* resonance of poly alanine in 80 % TFA–20 % $CDCl_3$. Different molecular weights and helix contents indicated.

block copolymers does not have free ends this indicates that the 'subsidiary' peaks in the homopolymer spectrum are largely due to end effects, since in the block copolymer there is little tendency for the L alanine residues near the ends of the PLA block to be predominantly in a 'coil' form. The rapid interconversion of helix and coil then covers the whole of the PLA block. One could also postulate that what is here termed the 'purely coil state' (characteristic also of poly DL alanine) is in fact a special coil structure that exists for times long on the n.m.r. scale due to a slow kinetic step in its formation. There is no independent evidence, however, for a special structure in low molecular weight PLA or in poly DL alanine.

That poly L alanine (and other polypeptides with hydrocarbon sidechains) interact with TFA in an unusual manner has been known for some while[29]

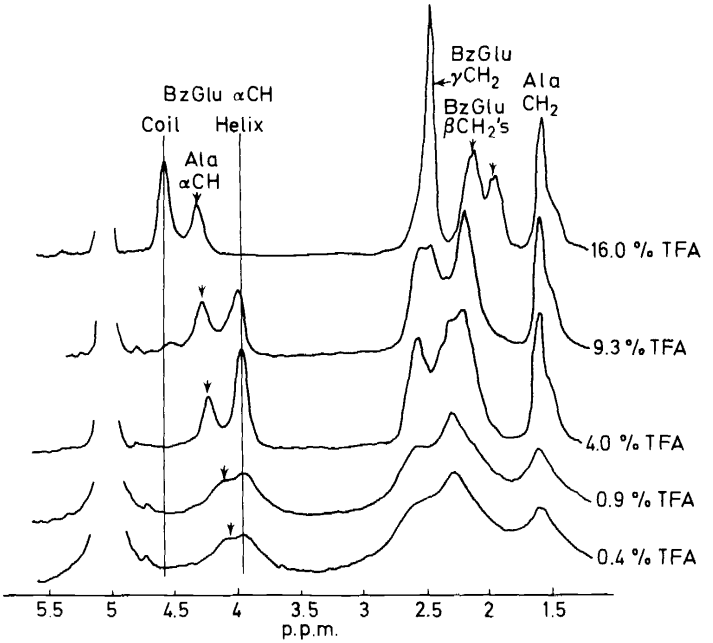



*Figure 14.* 220 MHz spectra in TFA–$CDCl_3$ of block copoly [benzyl L glutamate (39)–L alanine (46)–benzyl L glutamate (33)]. L Alanine αC*H* arrowed.

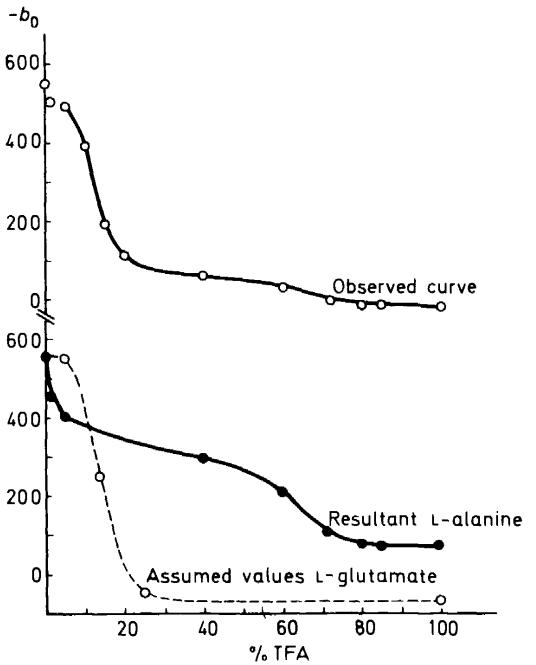



*Figure 15.* $b_0$ values in TFA–$CDCl_3$ for block copoly [benzyl L glutamate (39)–L alanine (46)–benzyl L glutamate (33)].

and *Figure 15* shows that the helicity of PLA in $CDCl_3$ is much reduced by the addition of very small amounts of TFA, unlike PBLG. In addition the infra-red spectra of high molecular weight PLA in $CDCl_3$–TFA show an unusual band at $\sim 1616\ cm^{-1}$ that grows as the TFA content is increased[29]. Since the amide I band at $\sim 1655\ cm^{-1}$ simultaneously decreases in intensity, the $1616\ cm^{-1}$ ('interaction') band can be assigned to amide I vibrations of polymer interacting with the acid. This assignment is supported by the fact that the interaction band has parallel dichroism when observed in oriented films of helical PLA. The band might alternatively be assigned to the asymmetric stretching of the charged carboxyl group of the trifluoroacetate ion if polymer protonation takes place. However, it has been observed[34] that the frequency of the interaction band remains constant if DCA is substituted for TFA, whilst the charged carboxyl band of these two acids differs by more than $20\ cm^{-1}$. Similar i.r. spectra have been observed for poly L leucine. In the case of poly L methionine and PBLG the 'interaction band' is also present in $CDCl_3$–TFA but is of low intensity. Furthermore, the appearance of the 'interaction band' is not accompanied by corresponding changes in amide II. It follows that there cannot be any large scale protonation of the amide group in polypeptides. The interaction is probably one of strong hydrogen bonding and a reduction in amide I frequency would be expected in these circumstances. It is difficult to establish whether this interaction is preferentially with the coil or the helix. The $1616\ cm^{-1}$ band is observed in films of helical poly alanine exposed to TFA or DCA vapour and moreover

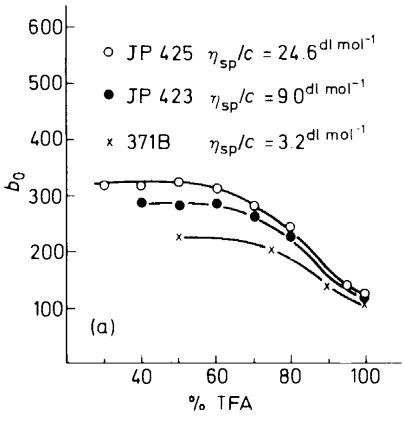



*Figure 16*(a). $b_0$ values in TFA–$CDCl_3$ for three poly D alanine samples of widely differing molecular weights.

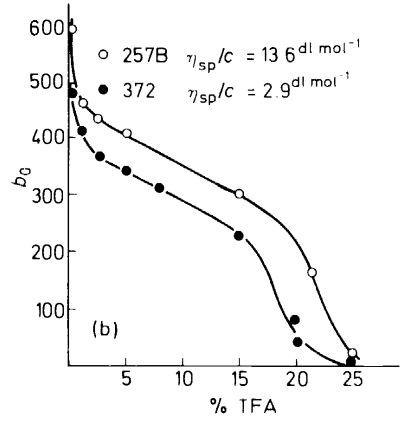



*Figure 16*(b). $b_0$ values in TFA–$CDCl_3$ for two samples of poly D α amino *n* butyric acid of widely differing molecular weights.

x-ray diffraction studies of such films[35] indicate order in the acid molecules and an overall increase in crystallinity with no apparent loss in polymer helicity. A structure has been proposed for the PLA α helix–acid complex[35]. Thus strong interaction of TFA with helical PLA is probably the cause of the

sharp changes in the PLA spectra (n.m.r. and i.r.) as small quantities of TFA are added to a $CDCl_3$ solution (*Figure 14*). In the random coil form of PLA the TFA probably also hydrogen bonds to the amide group since a broad amide I band results, giving a spectrum very similar to that of PBLG at high acid concentrations.

The 'single shifting' $\alpha CH$ peak for PLA (e.g. *Figure 11*) is in strong contrast to the 'double peak' observations for PBLG of similar molecular weight (e.g. *Figure 1*). We have compared by ORD the helix–coil transition of several PLA samples of widely differing molecular weights in the same way as described above for PBLG samples. There are unfortunately no molecular weight calibration data available that are appropriate for PLA and the polymers are characterized therefore only by their viscosities in DCA. Sample 371B [*Figure 16*(a)] is of very low molecular weight. The striking result is that there is very little dependence of helicity on molecular weight for poly alanine, in strong contrast to PBLG. Nowhere over the accessible solvent range does the $b_0$ differ by more than 50° for the two JP samples. The absence of any marked dependence of helicity on molecular weight is expected for a polypeptide showing a very broad helix–coil transition, i.e. one of low cooperativity and having only short helical segments. This means that in a polydisperse PLA sample there is only a narrow range of helicities present for a given solvent condition and therefore that rapid helix–coil interconversion gives rise to an essentially single peak.

## OTHER POLYPEPTIDES WITH HYDROCARBON SIDECHAINS

Poly D α-amino *n*-butyric acid has an ethyl sidechain and *Figure 16*(b) shows $b_0$ data for two samples of different molecular weight over the helix–coil transition. The molecular weight dependence of the transition is greater than for PLA but still much less than for PBLG and this is reflected in the n.m.r. spectrum shown in *Figure 17* for sample 372. Although the $\alpha CH$ resonance shows apparent multiplicity over the transition, the overall behaviour is intermediate between that of the 'single shifting peak' observed for PLA and that of the 'double peaks' seen in *Figure 1* for PBLG. This is emphasized by the fact that at the transition midpoint ($\sim$11 per cent TFA from the $b_0$ data) the $\alpha CH$ resonance does not look symmetrical: the centre of gravity of the resonance, however, does lie at the exact midpoint of the helix and coil shifts. Poly L leucine has been studied by several authors[9, 36] including ourselves and most samples have shown what approximates to a single shifting peak. A very low molecular weight sample studied by the Japanese authors, however, showed a quite distinct 'double peak' behaviour.

The general conclusion regarding $\alpha CH$ peak shapes to be drawn from these studies of polypeptides having hydrocarbon sidechains is as follows: for real samples, i.e. those having significant polydispersity, low cooperativity in the helix–coil transition means little dependence of helicity on molecular weight and a single shifting peak is normal for all but the very lowest molecular weights. A rise in transition cooperativity means the appearance of apparent multiplicity in the peak in the helix–coil transition region, particularly for low average molecular weights. For polymers having a highly cooperative transition the 'double peak' appearance will be typical and high average

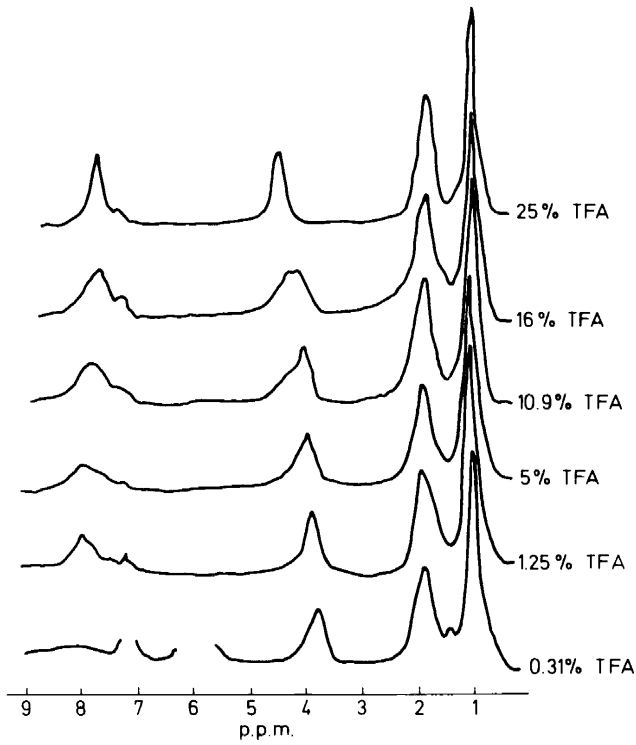



*Figure 17.* 100 MHz spectra of poly D α amino *n* butyric acid. Sample No. 372 in TFA–$CDCl_3$.

molecular weights must be achieved before all molecules in a sample have the same helicity and a single shifting α*CH* peak is observed.

## POLYPEPTIDES WITH AROMATIC SIDECHAINS

### Poly L tyrosine (PLT)

The study of the conformation of PLT in both aqueous and non aqueous solutions by ORD and CD has been subject to uncertainty due to the presence of sidechain chromophores overlapping those of the peptide groups. In aqueous solution a marked change occurs in the ORD/CD as the pH is lowered in the range 11.5 to 11.25 and this has been attributed to both a coil → helix[37] transition and to a coil → β-structure transition[38]. In most organic solvents PLT has been assumed to be helical. We have studied the n.m.r. spectrum of PLT[16] to obtain more information on the nature of the conformational transitions and to investigate the possibility that sidechain tyrosyl–tyrosyl interactions are responsible for the overlying Cotton effects in the ordered form. The spectrum of PLT in dimethylsulphoxide ($DMSOd_6$) exhibits relatively sharp resonances for all protons, including the α*CH* and amide N*H* suggesting a random coil structure. Addition of water to such

a solution causes a marked change in optical rotation between 5 and 10 per cent $D_2O$ and this is accompanied by changes in the n.m.r. spectrum (*Figure 18*). The αC*H* resonance moves upfield by about 0.3 p.p.m. over the range of 0 to 15 per cent $D_2O$ and thereafter remains constant in shift

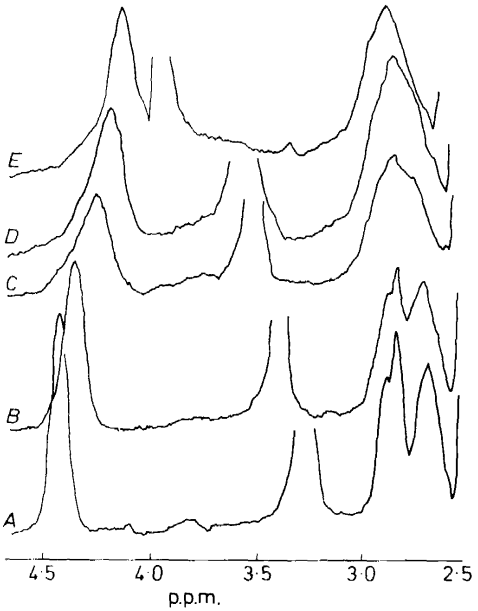



*Figure 18.* 220 MHz spectra of poly L tyrosine in $DMSOd_6$–$D_2O$ mixtures at 55°C. (A) pure $DMSOd_6$; (B) 3.9% $D_2O$; (C) 5.7% $D_2O$; (D) 6.7% $D_2O$; (E) 18.7% $D_2O$.

as more water is added. Some indication of αC*H* multiple-peak behaviour is seen in the middle of the transition and the changes have the character expected of a coil to helix change in a polymer of low cooperativity. Some broadening of the aromatic protons also takes place over the same solvent range with little change in chemical shift. There is strong evidence that PLT is helical in trimethylphosphate (TMP)[39,40] and addition of TMP to a solution of PLT in $DMSOd_6$ causes a sharp change in the ORD at ~35 per cent TMP, similar to that observed on $D_2O$ addition. *Figure 19* shows the changes in the aromatic spectrum and as with $D_2O$ addition there is considerable broadening with little change in chemical shift. Thus there is a strong indication that PLT is random coil in DMSO and that there is transition on $D_2O$ or TMP addition to a more rigid conformation, probably the α-helix. However, there are no marked changes in aromatic chemical shifts as would indicate strong tyrosyl–tyrosyl interactions.

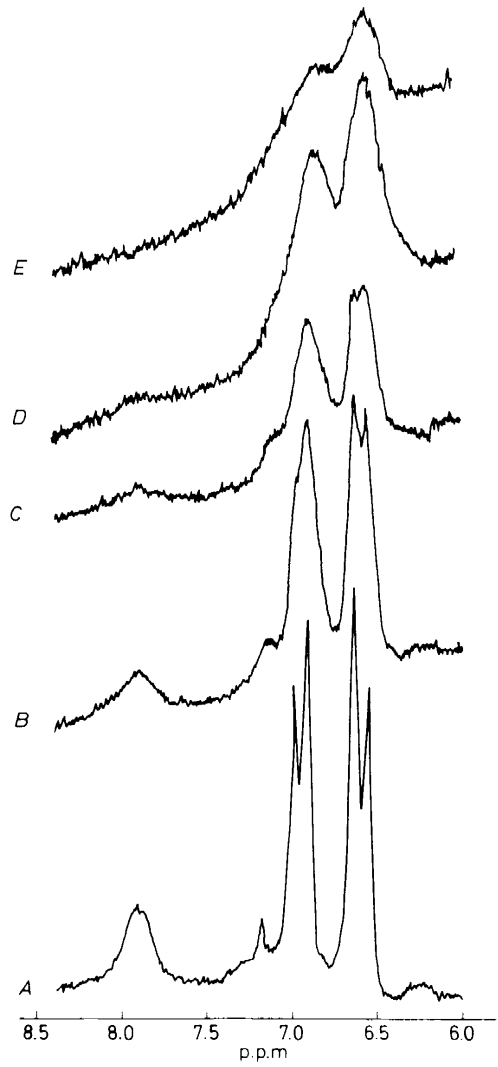



*Figure 19.* 100 MHz spectrum of poly L tyrosine in $DMSOd_6$–TMP at 29°C. %TMP: (A) 0; (B) 2.0; (C) 2.5; (D) 30; (E) 60.

**Poly L aspartate esters**

The conformations of ester derivatives of poly L aspartic acid have been shown to be dependent on the precise nature of the sidechain and on the solution conditions[41]. Thus whereas poly β benzyl L aspartate and poly β methyl L aspartate take up the left handed (LH) helical conformation in chloroform the β ethyl, β propyl and β phenethyl esters are in the right handed (RH) helical form. Furthermore, copolymers of a LH-supporting

sidechain, e.g. benzyl, with a RH-supporting sidechain, e.g. ethyl, undergo transition from the RH helical form to the LH helical form as the temperature is raised. The temperature at the midpoint of the transition is dependent on the copolymer composition. This delicate balance of helical senses and the relative ease with which either helix sense can be broken down to the coil by means of haloacetic acids indicate that the intramolecular forces differ markedly from those in such polypeptides as PBLG. Scheraga and his co-workers[42] have calculated the sidechain conformations corresponding to minimum energy of both helix senses of several poly L aspartates. They find four such conformations, two longitudinal and two transverse with respect to the helix direction. The preferred helix sense for each polymer follows from these calculations and usually is found to agree with that observed. The n.m.r. spectra of these polymers have been studied[43] to follow the variation in helix sense of L-aspartates (using the shift of the two main chain protons) and also to investigate the proposals[42] of specific sidechain conformations (using principally the spectrum of the $\beta CH_2$ group).

## MAIN CHAIN RESONANCES

The effect of helix sense on the n.m.r. spectrum can be readily investigated by inclusion of L alanine residues in poly β benzyl L aspartate since as little as ten per cent L alanine is sufficient to swing the helix sense from LH to RH. *Figure 20* shows the spectrum of both helix senses in $CDCl_3$ (the 0.5 per cent

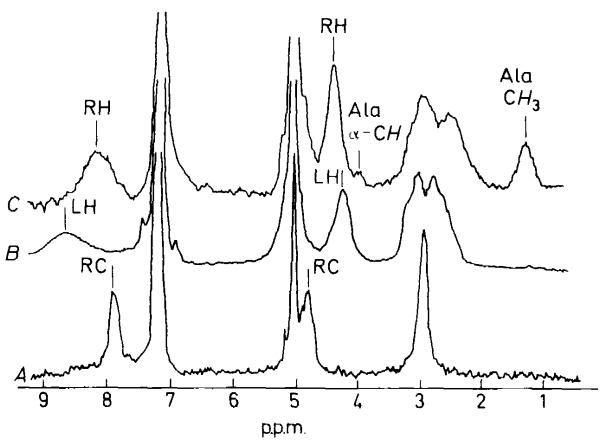



*Figure 20.* 100 MHz spectra of poly β benzyl L aspartate: (a) random coil (RC) form in 5% TFA–95% $CDCl_3$; (b) left handed (LH) helical form in 0.5% TFA–99.5% $CDCl_3$; (c) right handed (RH) helical form of poly [β benzyl L aspartate (90)–L alanine (10)] in 0.5% TFA–99.5% $CDCl_3$.

TFA is added to avoid aggregation broadening) and the random coil in $CDCl_3$–5 per cent TFA. The amide N*H* resonances is seen to be markedly dependent on conformation[56]. For PBLA the shift values from internal TMS are as follows: coil–8.00 p.p.m. (PBLG coil 7.95 p.p.m.), LH helix—8.75 p.p.m.,

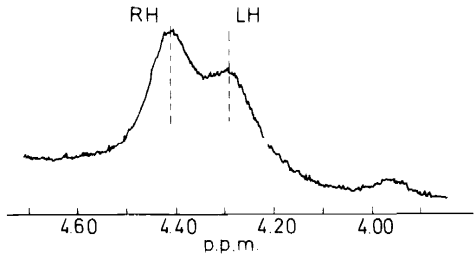



*Figure 21.* 300 MHz spectrum of the αC*H* resonance of helical poly [β benzyl L aspartate (95)–L alanine (5)] in 0.5% TFA-99.5% $CDCl_3$.

RH helix—8.20 p.p.m. (PBLG helix—8.17 p.p.m.). Thus although the shifts for the coil and RH helix and thus their difference, are similar to those of PBLG, the LH helix is at markedly lower field. The shift difference between the two helix senses is thus 0.55 p.p.m. for this resonance.

The PBLA αC*H* shift is also dependent on conformation; 4.85 p.p.m. for the coil (PBLG coil 4.45 p.p.m.), LH helix—4.30 p.p.m. and RH helix—4.40 p.p.m. (PBLG helix—3.95 p.p.m.). For this main chain proton therefore, neither the RH helix nor the coil shifts are similar to those of PBLG, although the RH helix–coil shift difference $\Delta_{H/C}$ is the same for both polymers. The αC*H* shift difference between the two helix senses is thus 0.10 p.p.m. and though small, the coexistence of both helix senses can be observed in a suitable polymer. This is seen in *Figure 21* for a PBLA–5 per cent L alanine copolymer in $CDCl_3$–0.5 per cent TFA. The L alanine αC*H* peak is seen at 3.96 p.p.m., a value characteristic of RH helical polypeptides such as PBLG in $CDCl_3$–TFA.

It is important to know whether the unusual shift values for the poly L aspartate main chain protons are general to all poly L aspartates. We have observed the spectrum of helical poly β methyl L aspartate (LH) in $CDCl_3$–0.5 per cent TFA and the N*H* resonance was observed at 8.73 p.p.m. The αC*H* resonance has previously been reported at 4.27 p.p.m.[9]. Poly β ethyl L aspartate (RH) under identical helix-promoting conditions showed an N*H* peak at 8.16 p.p.m. and an αC*H* peak at 4.4 p.p.m. The shift differences between the two helix senses are thus general to poly L aspartate esters.

## THE SIDECHAIN SPECTRUM

The sidechain spectra of poly L aspartates particularly that of PBLA in the LH helical form have been analysed[43] in terms of both shifts and coupling constants, largely with a view to establishing whether in the helical form the sidechain is at all rigidly held in preferred conformations.

*Figure 20* (at 100 MHz) shows that in the coil form of PBLA the two β protons are roughly equivalent, as are the two benzyl protons. Spectra at 220 MHz reveal small shift differences (and hence essentially AB quartets) for both pairs: $\Delta \approx 0.07$ p.p.m. ($\beta CH_2$) and $\Delta \approx 0.05$ p.p.m. (benzyl $CH_2$). Such small differences are not unexpected, bearing in mind the presence of the

α asymmetric centre, and do not indicate anything unusual in the random coil conformation.

In the LH helical form of PBLA (*Figure 20*) the shift difference $\Delta$ between the two β protons rises to 0.37 p.p.m.[56] whilst in the RH helical form containing 10 per cent L alanine the difference lies between 0.5 and 0.6 p.p.m. Poly β ethyl L aspartate (RH helix) shows a $\beta CH_2$ shift difference of ~0.6 p.p.m. and a dependence of this difference on helix sense seems general. As with the helix sense dependence of the main chain protons, this sidechain shift parameter is clearly of diagnostic value for helix sense, but cannot readily be used to obtain more detailed structural information. The measurement

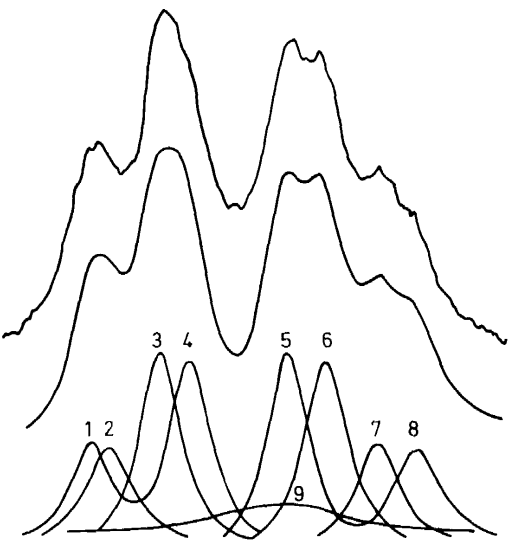



*Figure 22.* 100 MHz spectrum of the $\beta CH_2$ group of poly β benzyl L aspartate in $CDCl_3$ at 100°C together with a curve resolver readout and analysis.

of coupling constants, however, can in principle be made to yield detailed structural parameters. Although very difficult to observe with polypeptides in the helical form, due to the rather broad lines, we have had some success at measuring $J$ values with PBLA at elevated temperatures. *Figure 22* shows the $\beta CH_2$ resonance at 100°C in pure $CDCl_3$[43]. It is an ABX system and the couplings to the X proton ($\alpha CH$) are just visible, amounting to $J_{\alpha,A} = 4 \pm 1$ Hz, $J_{\alpha,B} = 7.4 \pm 0.3$ Hz. The three possible staggered conformations about the α—β bond can be represented as in *Figure 23* and we have attempted a conformational analysis about this bond using $J_{trans} = 13.6$ Hz and $J_{gauche} = 2.6$ Hz. Using the usual equations the three relative lifetimes are found to be $a_1 = 0.14$, $a_2 = 0.39$ and $a_3 = 0.47$, although there is the alternative solution with $a_1$ and $a_3$ interchanged, since the two β protons cannot be unequivocally assigned. Inspection of molecular models, however,

suggests that rotamer 2 cannot have a significant population due to steric hindrance between the sidechain carboxyl group and the helical backbone. If $a_2 = 0$, then $J_{\alpha A} + J_{\alpha B} = J_g + J_t$. Since this is not found one is led to conclude that the regular 60° rotamers of *Figure 23* are not appropriate. Inspection of the space filling model indicates that for rotamer one the $C_\alpha H_X$—$C_\beta H_B$ angle might increase by about 10° and for rotamer three the $C_\alpha H_X$—$C_\beta H_A$ angle might increase by about 15°. The application of a Karplus type function for the angular variation of the coupling constant to these

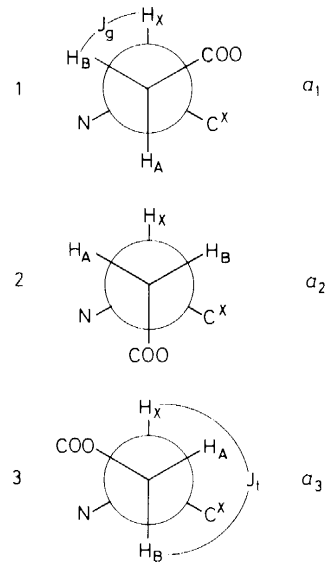



*Figure 23.* The three possible staggered conformations about the α—β bond of poly (aspartate esters).

'distorted' rotamers leads to an effective *trans* coupling constant of 11 to 12 Hz and an effective *gauche* coupling constant of 0.5 to 1 Hz. The sum of these quantities is not far from the sum of the observed couplings and an analysis with these constants suggests a 2:1 ratio of the 'distorted' rotamers (without of course being able to distinguish which predominates). In comparing these results with the preferred conformations of Scheraga and co-workers[42], the first conclusion that can be drawn simply by a comparison of the measured vicinal coupling constants with the postulated $\cos^2$ variation of $J$ with α—β bond angle, is that no single rotamer can possibly satisfy the observed $J$ values. In both the predicted LL(−) conformation (having a transverse sidechain arrangement) and in the LL(+) (having a longitudinal sidechain arrangement), the $\beta CH_2$ conformation approximates to that of rotamer 1. The precise bond angles indicate that one β proton should show a vicinal coupling of 12 13 Hz and the other a coupling of 1–3.5 Hz. These values are well outside the experimental error and reinforce the conclusion that under the

experimental conditions the $\beta CH_2$ is in rapid motion between two well separated conformers, both having a significant lifetime. This result is not compatible with the degree of sidechain immobilization envisaged in the calculations of helix sense since the presence of a dominant sidechain conformation would certainly have been apparent from the $\beta CH_2$ spectrum.

The benzyl $CH_2$ spectrum of LH helical PBLA is a distinct AB quartet with $\Delta = 0.13$ p.p.m. Introduction of ten per cent L alanine induces transition to the RH form and the shift difference $\Delta$ changes to 0.28 p.p.m. The complete data on this shift difference are given in *Figure 24* together with $b_0$ data to indicate change of helix sense. The $\Delta$ values in both helices are much in excess of those in the coil and since the benzyl $CH_2$ group of PBLG under similar solvent conditions shows no measurable splitting at 220 MHz it follows that the asymmetry of the L aspartate helix is strongly felt at the benzyl

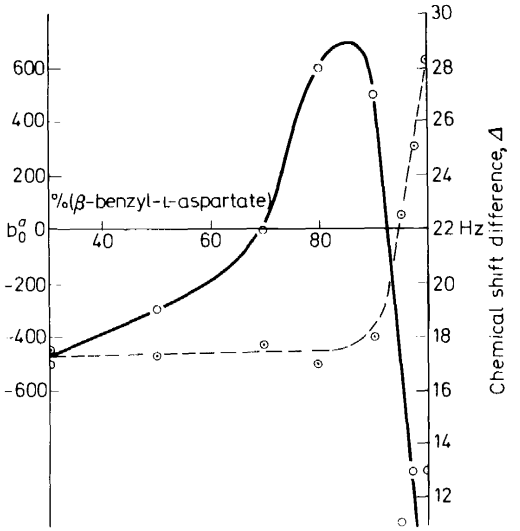



*Figure 24.* The shift difference $\Delta$ (at 100 MHz) between the benzyl $CH_2$ protons of β benzyl L aspartate residues and $b_0$ for the series poly [benzyl L aspartate–L alanine] in TFA–$CDCl_3$ at room temperature. Full line: shift difference; broken line: $b_0$.

$CH_2$ group. It is impossible, however, to obtain any estimate from these $\Delta$ values of the degree to which the sidechain is restricted at the $\beta CH_2$ group. Large shift differences between the benzyl protons could be generated by preferred conformations with respect to the benzene ring. However, the $J_{gem}$ coupling of the benzyl $CH_2$ group in LH helical PBLA is the same as that of the random coil form and that of the monomer; this does not suggest any change in the conformation about this bond on forming the helix.

## CONFORMATIONS OF ASPARTATE COPOLYMERS WITH GLUTAMATES

The $\alpha CH$ and amide $NH$ chemical shifts of poly aspartate esters are

both dependent on helix sense and well separated from those of poly benzyl glutamate esters. This allows separate observation of the two types of residue in block and random copolymers and thus a determination of their conformation. Such an n.m.r. approach avoids reliance on a parameter such as $b_0$ that gives simply a sum of the conformations of both types of residue. Several series of A—B block copolymers (where A represents PBLA and B represents PBLG) were studied in $CDCl_3$–TFA[44]. In polymer Series 455 the L aspartate block was synthesized first, being held constant at an estimated 100 residues, and the L glutamate block of varying length was then added on without isolation of the first block. In Series 449 the order was reversed, the L gluta-

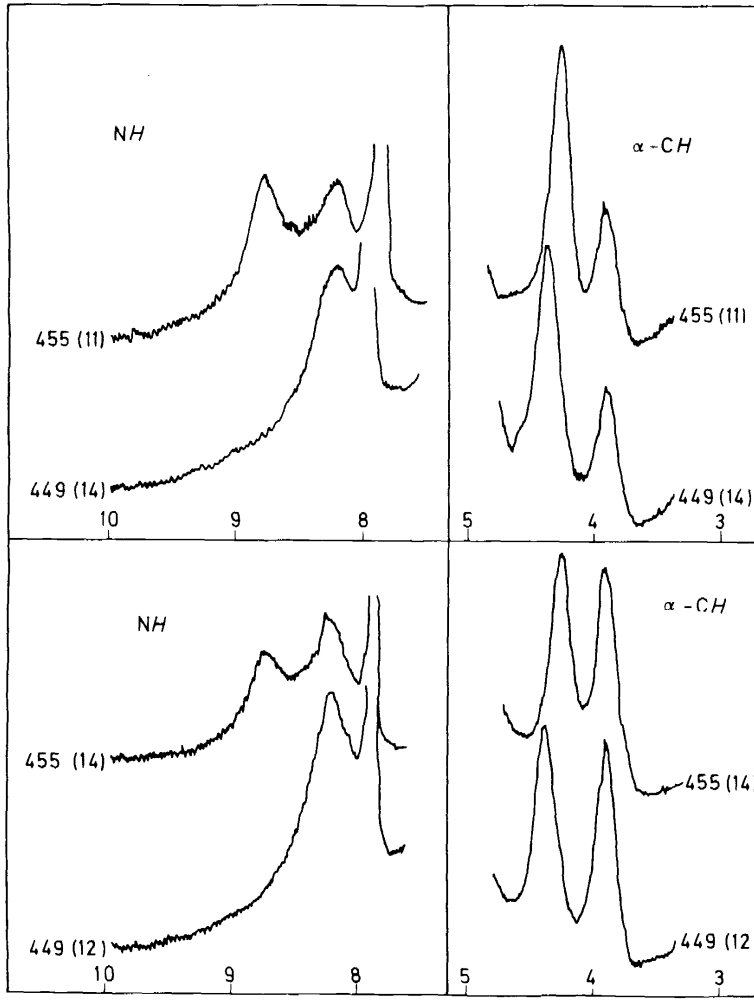



*Figure 25.* 220 MHz spectra of α*CH* and N*H* regions of helical block copolymers (in TFA–$CDCl_3$). Series 455 (left-handed aspartate) No. 11 (100 aspartate: 40 glutamate), No. 14 (100 aspartate: 100 glutamate); Series 449 (right-handed aspartate) No. 14 (200 aspartate: 100 glutamate) and No. 12 (100 aspartate: 100 glutamate).

mate block being synthesized first. *Figure 25* shows spectra of two fully helical polymers from both series. From the chemical shift data presented above the L aspartate αC*H* shift of 4.30 p.p.m. and the amide N*H* shift of 8.80 p.p.m. in Series 455 polymers indicates a LH helical poly β benzyl L aspartate block. This is the expected conclusion since PBLA normally takes up the LH helix. In Series 449 however, the L aspartate αC*H* is at 4.40 p.p.m. and the amide N*H* at ~8.2 p.p.m.: this indicates a RH helical poly β benzyl L aspartate block. The most reasonable explanation for this is that there is a certain degree of overlap from the first synthesized block into the second block (as a result of some unreacted monomer still remaining). For Series 455

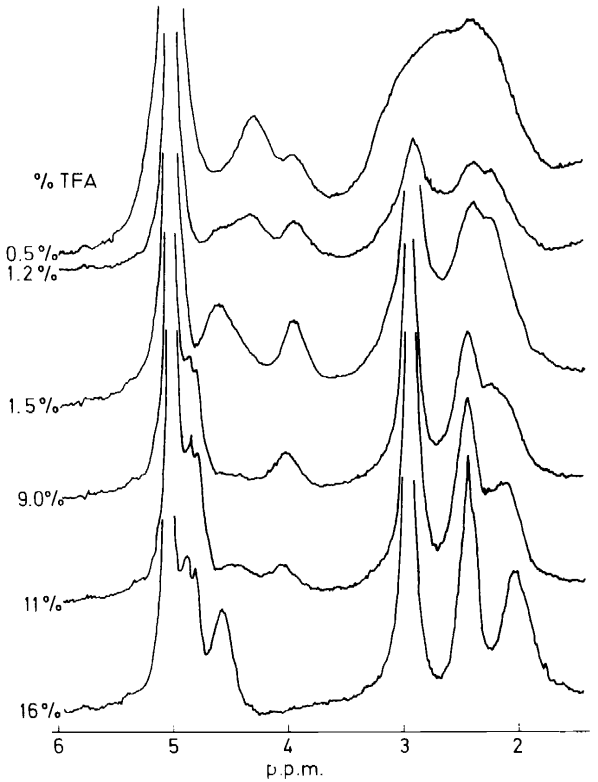



*Figure 26.* 100 MHz spectra, 1–6 p.p.m., of 450 (No. 14) (100 glutamate: 150 aspartate) in TFA–$CDCl_3$. Assignments: 2.1 p.p.m. glutamate β$CH_2$; 2.4 p.p.m. glutamate γ$CH_2$; 3 p.p.m. aspartate β$CH_2$; 4–5 p.p.m. α*CH*s and 5.05 p.p.m. benzyl $CH_2$s.

this means aspartate in the L glutamate block, but an L aspartate block free of L glutamate and therefore left handed. The reverse is true for Series 449 in that the L glutamate can overlap into the L aspartate block. Since ~15 per cent PBLG introduced into PBLA can cause a reversal of the helix sense from LH to RH[45], it is understood why the PBLA block of Series 449 is right handed.

The L glutamate monomer (as *N*-carboxyanhydride) cannot therefore have reacted to completion before the L aspartate monomer was added. A further Series (No. 450) was studied in which the L glutamate block of standard length was synthesized first and the length of the L aspartate block was increased over a wide range. When the L aspartate block was short it was found to be RH helical but as the length of the block increased the proportion of LH helix increased as judged by the $\alpha CH$ spectrum. This is understandable if the amount of unreacted glutamate remaining at the moment of addition of L aspartate *N*-carboxyanhydride were constant for all polymers since the percentage L glutamate overlapping into the L aspartate block would then be at a maximum for the shortest L aspartate block and decrease as the length of the L aspartate block increases. With a significant degree of overlap in many copolymers it can be asked whether they may be truly regarded as block copolymers. This can be assessed by gradual addition of TFA to a $CDCl_3$ solution and observing separately and simultaneously the helix to coil transition of the L aspartate and L glutamate residues. From the $\alpha CH$ spectrum in *Figure 26* of a Series 450 copolymer having a considerable degree of overlap it can be seen that the two residues titrate quite separately. At 1.2 per cent TFA the L aspartate block is in the middle of its transition, which is nearly complete at 1.5 per cent TFA. The L glutamate transition has not started at 9 per cent TFA and has a midpoint at 11 per cent TFA. Both blocks are coil in 16 per cent TFA. It follows that the polymer is a genuine two-block copolymer and the degree of overlap although significant, is not excessive.

We have also studied two series of similar random copolymers[46]: benzyl L aspartate copolymerized with benzyl D glutamate (Series 441) and with benzyl L glutamate (Series 432). In the first case both residues favour the same helix sense (LH) whilst in the second they favour the opposite sense. Titration of these copolymers against gradually increasing amounts of TFA in $CDCl_3$ permits the helix to coil transition of the L aspartate and L or D glutamate residues to be separately followed using the $\alpha CH$ spectrum. As with the block copolymers this is a consequence of the fact that a 'double peak' behaviour is observed for both constituents. The helix–coil breakdown can be characterized by the transition midpoint as judged by equal areas for the helix and the coil component peaks of the $\alpha CH$ resonance. In contrast to the block copolymers, the transition midpoints of both constituents fell in the same range of TFA concentration, as expected for random copolymers. In the case of the copolymers of $\beta$ benzyl L aspartate with $\gamma$ benzyl D glutamate (Series 441) having LH helices throughout, no difference could be detected in the transition midpoints of the two constituents and the value fell approximately linearly from 10 per cent TFA for homopoly $\gamma$ benzyl D glutamate to 1.5 per cent TFA for homopoly $\beta$ benzyl L aspartate. In the case of Series 432 there is a change of helix sense from LH to RH as more than 10 per cent L glutamate is added to PBLA. This is readily seen from the shift of the L aspartate $\alpha$CH as explained above. The transition midpoints for Series 432 are plotted in *Figure 27* and the helix stability is seen to pass through a minimum at the point (10 per cent L glutamate) at which there is a balance between the two helix senses. Furthermore in the 50–50 copolymer there is a distinct difference between the transition midpoints of the two components.

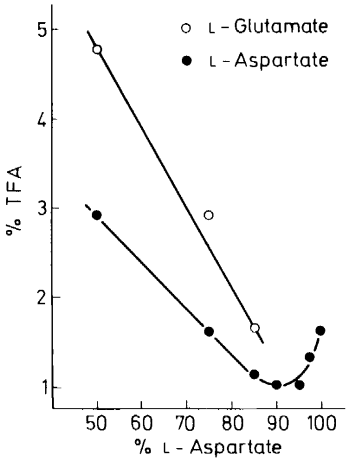



*Figure 27.* Helix–coil transition mid-points of Series 432: copoly [γ benzyl L glutamate, β-benzyl L aspartate].

This is interpreted as resulting from irregular distribution of the component residues along the chain, i.e. homopolymeric runs occur, longer than would be expected for truly random polymerization.

**DL Copolymers**

The conformations of racemic copolymers of random sequence have long been the subject of study, due largely to the inability of ORD/CD to provide a clear-cut answer. The intensity of the amide V band characteristic of helical polypeptides allowed[47] Tsuboi *et al.* to suggest that in the solid state PBDLG is about one half helical. The solution conformation cannot, however, be approached by this method due to the lack of suitable solvents but the α*CH* region of the n.m.r. spectrum is a direct and versatile experimental method. Bovey *et al.*[48] showed that a PBDLG sample in chloroform has an α*CH* peak at 3.95 p.p.m. (a value characteristic of helix) and moreover on TFA addition shows the 'double-peak' spectrum typical for the helix–coil transition of a low molecular weight PBLG sample. It was concluded that in chloroform the polymer was helical.

In a recent paper Paolillo *et al.*[49] have used the α*CH* spectrum of a number of DL copolymers to investigate solution conformations in several solvents. In chloroform PBDLG samples having a DP greater than about 150 were found to be fully helical, whilst the lowest molecular weight studied (DP ≈ 17) was less than half helical. The shift of the coil α*CH* was shown to be about 0.3 p.p.m. to lowfield of the helix. In dimethylformamide (DMF) the α*CH* showed a shift difference of 0.30 p.p.m. (helix shift 4.09 p.p.m., coil shift 4.39 p.p.m.) and whilst the PBDLG of DP = 170 (375 (1)) was fully helical, that of DP ≈ 17 (400A) was almost fully coil. *Figure 28* shows the results obtained for several PBDLG samples together with the corresponding spectrum of helical PBLG. In dimethylsulphoxide (DMSO) which

is a poorer promoter of the helical conformation than DMF (although PBLG is helical in DMSO), all the PB*DL*G copolymers were found to be in the random coil. The helicity of racemic PB*DL*G copolymers in solution was therefore shown to depend both on the solvent and on the molecular weight.

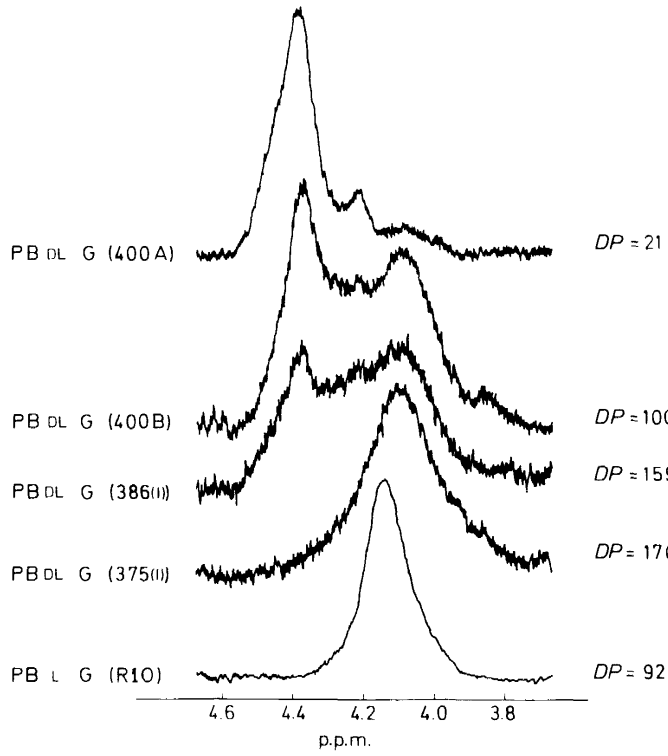



*Figure 28.* 220 MHz $\alpha CH$ spectra in DMF of four samples of poly γ benzyl DL glutamate having differing molecular weights and one sample of poly γ benzyl L glutamate.

The same paper also considered the conformation in chloroform of racemic copolymers of poly β benzyl aspartate (PBDLA) and of the methyl ester (PMDLA). L Aspartate residues on LH helices (which is the natural sense for both the benzyl and methyl esters) give rise to a peptide N*H* peak at 8.75 p.p.m.[43], i.e. well separated from that of the RH helix or coil conformations at 8.2–8.3 p.p.m. The N*H* region proved therefore to be of the greater diagnostic value. A PBDLA sample of DP ~60 showed an N*H* peak at 8.75 p.p.m. having less than half the total intensity of the N*H* resonance region. It was concluded that this sample is more than half in the coiled conformation. This was confirmed by the fact that on TFA addition the $\alpha CH$ resonance showed no sign of the double-peak phenomenon and moved downfield as a single line. By the same criteria as these, the sample of PMDLA (DP ~140) studied was concluded as being fully coil.

## $^{13}$C SPECTROSCOPY OF SYNTHETIC POLYPEPTIDES

Preliminary investigations of the helix–coil transition of PBLG have been made by FT spectroscopy at natural abundance[50]. As with many such polymer studies it was hoped that in comparison with proton spectra the lines would be very much sharper and the dependence of chemical shifts on configurational and conformational differences would be very much greater. *Figure 29* shows proton decoupled $^{13}$C/FT spectra of 15% w/v solutions of

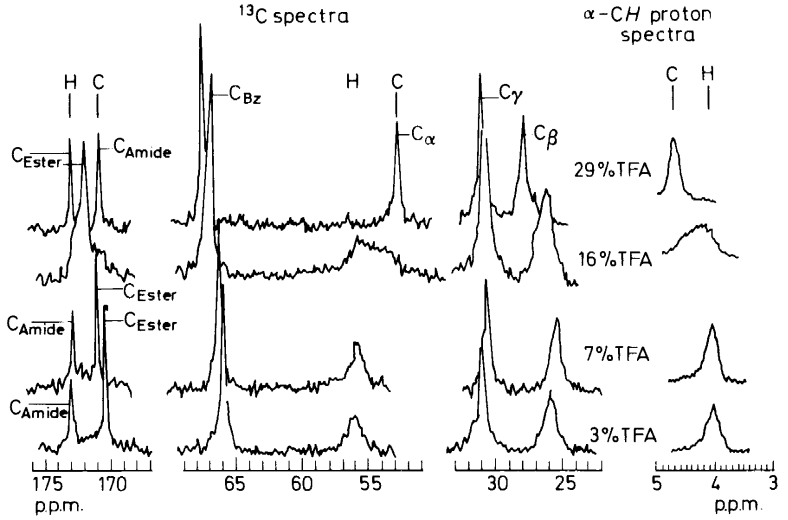



*Figure 29.* $^{13}$C spectra at 25.2 MHz and $^1$H spectra at 100 MHz of poly (γ benzyl L glutamate) in TFA $CDCl_3$: 3% and 7% TFA—helix; 16% TFA—helix plus coil; 29% TFA — coil. H, helix; C, coil shifts for $C_{amide}$, $C_\alpha$ and α*CH* resonances.

PBLG in $CDCl_3$–TFA solvents and *Table 2* gives the observed shifts for the coil form in 29 per cent TFA and the helix form in 3 per cent TFA.

Several striking changes in chemical shift take place as the TFA concentration increases and helix is converted into coil. The α carbon moves upfield by 3.5 p.p.m. over the transition and in the intermediate region (16 per cent TFA) shows a multiple peak behaviour very similar to that seen in the proton spectrum.

It has been shown above that this is a consequence of end effects and polydispersity. The other main chain carbon atom, the amide carbonyl, likewise shows an upfield shift, of 2.5 p.p.m. in this case. The β carbon moves downfield by about 1 p.p.m. over the transition and the γ carbon remains approximately constant in shift. These displacements appear to be a direct consequence of the conformational transition since change of TFA concentration between three and seven per cent has no effect on the peak positions. This is in contrast to the benzyl carbon and the ester carbonyl resonances that show a monotonic displacement downfield with increasing

TFA from the very first addition. This is clearly due to solvation of the ester group by TFA, presumably by hydrogen bonding to the carbonyl group. Solvation of the ester carbonyl group by TFA results in a downfield shift of about 2 p.p.m. and if a similar value is appropriate to solvation of the

*Table 2.* $^{13}C$ Chemical shifts in p.p.m. from internal $Me_4Si$ of poly (γ benzyl L glutamate) solutions in chloroform–trifluoroacetic acid

| Solvent | | $C_\beta$ | $C_\gamma$ | $C_\alpha$ | $C_{BZ}$ |
|---|---|---|---|---|---|
| 3% TFA<br>97% $CDCl_3$<br>(15% PBLG) | HELIX | 25.9 | 30.7 | 56.3 | 65.9 |
| 29% TFA<br>71% $CDCl_3$<br>(15% PBLG) | COIL | 27.1 | 30.6 | 53.2 | 67.6 |
| Solvent | | $C_{2,6}$ – Ar | $C_1$ – Ar | C=O (Ester) | C=O (Amide) |
| 3% TFA<br>97% $CDCl_3$<br>(15% PBLG) | HELIX | 126.6 | 133.6 | 170.5 | 173.1 |
| 29% TFA<br>71% $CDCl_3$<br>(15% PBLG) | COIL | 126.6<br>127.1 | 133.6 | 173.2 | 171.1 |

amide carbonyl under the same solvent conditions, then an upfield displacement of the amide carbonyl over the helix–coil transition could be intrinsic to the conformational change. It is not possible on the above data to assess whether the upfield α carbon displacement is intrinsic to the conformational difference since the effects of TFA solvation on this peak are not defined.

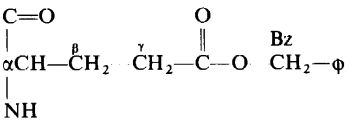
Comparison of the proton spectra of PBLG (e.g. *Figure 1*) with the $^{13}C$ spectra of *Figure 29* reveals an unexpected overall similarity, particularly when comparing the α carbon and α hydrogen peaks. Thus the α carbon widths (~65 Hz for the helix and ~17 Hz for the coil) are not dramatically less than peak displacements due to the conformational change from coil to helix (~76 Hz). Since the helix and coil are ~50 Hz apart in the α*CH* proton spectrum at the same applied field, the α carbon spectrum cannot provide substantially new information on exchange rates between the helix and coil conformations. Although the situation would be somewhat improved by working at higher fields the relatively broad lines in the carbon spectrum will make it difficult to distinguish small structural differences (as has been done for small organic molecules in solution) or to study proteins in large rigid conformations with much greater ease than is possible with the proton spectrum. The carbonyl resonances are much sharper than the α carbon and thus of potentially greater value, though often the presence of additional peaks in this region complicates the issue. Carbonyl peaks have the disadvantage of no intensity enhancement in proton decoupled spectra. Similar results to our own were subsequently reported by Boccalon *et al.*[51] on poly *N* δ carbobenzoxy L ornithine in $CDCl_3$–TFA, the main chain carbons showing similar displacements over the helix–coil transition as PBLG. The side-

chain urethane carbonyl is markedly upfield of the amide carbonyl in this polymer and shows downfield solvation shifts of similar magnitude to those of the PBLG ester carbonyl.

We have also studied the $^{13}C$ spectrum of poly γ benzyl L glutamate in DMSO to observe the helical shifts and the racemic PB*DL*G to observe the coil shifts. Great similarity to the $CDCl_3$–TFA spectra was found viz. the helix αC peak was 4.5 p.p.m. to low field of the coil, the helix amide carbonyl was 4.2 p.p.m. to low field of the coil and the helix β carbon was 1.6 p.p.m. upfield of the coil.

Measurements have also been made of $T_1$ values for the PBLG carbon atoms under three solvent conditions in $CDCl_3$–TFA (corresponding to aggregated helix, to non-aggregated helix and to coil), using the 180°–τ–90° pulse sequence. The results, together with estimates of $T_2$ from linewidths are given in

*Table 3.* $^{13}C$ relaxation times in seconds, for poly γ benzyl L glutamate (30% solution in $CDCl_3$–TFA)



| | Aggregated helix (3% TFA) | | Helix (12% TFA) | | Coil (50% TFA) | |
|---|---|---|---|---|---|---|
| | $T_1$* | $T_2$* | $T_1$ | $T_2$ | $T_1$ | $T_2$ |
| αC | ~0.03 | <0.003 | 0.1 | 0.005 | 0.06 | 0.02 |
| βC | ~0.03 | <0.003 | 0.05 | 0.007 | 0.04 | 0.01 |
| γC | ~0.03 | ~0.003 | 0.05 | 0.01 | 0.05 | 0.02 |
| BzC | 0.1 | ~0.005 | 0.1 | 0.02 | 0.1 | 0.04 |
| $\phi C_1$ | 3.3 | | 3.0 | | 3.6 | |
| $\phi C_2$–$C_6$ | 0.8 | | 0.6 | | 2 | |
| Amide C=O | 0.9 | ~0.005 | 0.9 | 0.02 | 0.7 | 0.03 |
| Ester C=O | 1.9 | | 1.4 | 0.03 | 1.6 | 0.05 |

*$T_1$ from 180–τ–90 sequence: $T_2$ from linewidth.

*Table 3.* The $T_2$ results are rough and moreover maximum values, since apparent linewidths will exceed true linewidths due both to the presence of structural heterogeneity and to the use of a transform of finite length. Nevertheless, the $T_2$ values are always less than $T_1$, particularly for the main chain carbons. $T_1$ remains roughly constant as the conformation changes due to more TFA addition, whilst $T_2$ values increase. This would suggest that correlation times must be of the order of a Larmor period, i.e. in the region of the $T_1$ minimum. Careful checking however, by measurements of $T_1$ at different applied fields, is required before any firm statements about correlation times can be made.

Several poly L aspartate polymers and copolymers in $CDCl_3$–TFA have been studied to investigate the dependence of the α carbon shift on helix sense and to discover whether the L aspartate shifts differ from those of the corresponding L glutamates, as found in the proton spectra. The polymers used

(in addition to PBLG) were PBLA and copolymers of PBLA with PBDG (LH helical L aspartate) and with PBLG (RH helical L aspartate). The absolute magnitudes of the α carbon L aspartate shifts differ from those of the L glutamate and moreover there is a dependence on helix sense. The results are summarized in *Table 4* in p.p.m. from internal TMS.

*Table 4*

| | | αC shift | | | αC shift |
|---|---|---|---|---|---|
| PBLG | Helix | 57.3 | PBLA | LH helix | 51.3 |
| PBLG | Coil | 54.3 | PBLA | Coil | 50.6 |
| PBLA | RH helix | 53.7 | | | |

It is seen that although the L aspartate shifts are all upfield of the L glutamate, the difference between RH L aspartate helix and coil (3.1 p.p.m.) is close to the difference between L glutamate helix and coil (3.0 p.p.m.), the coil being upfield of the helix in both cases. In both the peptide N*H* and α*CH* proton spectra the helix–coil differences $\Delta_{H/C}$ are similar for RH L aspartates and RH L glutamates, despite differences in absolute shift (see *Table 1*). The LH L aspartate helix gives a very anomalous N*H* shift, however. In the α carbon spectrum the LH L aspartate shift is only 1.4 p.p.m. upfield of the RH L aspartate α carbon and the LH helix–coil difference is thus reduced to 0.7 p.p.m. The LH L aspartate helix appears anomalous therefore in both the $^{1}H$ and $^{13}C$ spectra. These differences in the carbon spectrum between glutamates and aspartates (*Table 4*) have potential uses in conformational analysis.

## $^{13}C$ SPECTRA OF POLYPEPTIDES IN WATER

It is of great interest to study water-soluble polypeptides in order to establish whether there are any peak displacements that may be reliably correlated with conformation and therefore valuable in the study of protein conformation in solution. To this end we have obtained the $^{13}C$ spectrum of copoly (L glutamic acid[42], L lysine[28], L alanine[30]). We have previously studied the proton spectrum[5,11] and ORD of this polymer, which shows a lesser tendency to aggregation on helix formation than is shown by homopoly L glutamic acid or homopoly L lysine. Nevertheless, the α carbon resonance of the 60 to 70 per cent helical polymer at pH 2.5 had a width of some 80 Hz. As the helicity was changed from ~70 to ~10 per cent the amide carbonyl was displaced upfield by ~2.4 p.p.m. and the α carbon upfield by ~2·0 p.p.m. Both these values are approximate due to the fact that both peaks appeared multiple, perhaps due to differences between the component amino acids. In terms of a complete helix to coil change these differences would amount to about 3.5 p.p.m. for the amide carbonyl and about 3.0 p.p.m. for the α carbon. Recently the helix–coil transition of poly L glutamic acid has been studied as a function of pH in water by Lyerla *et al.*[52]. They were able to make measurements down to pH 4.6, at which the polymer was about three quarters

helical, below which aggregation broadening precluded observations. As the pH was lowered from neutral to 4.6, both the α carbon and the amide carbonyl resonances were displaced about 2 p.p.m. downfield. In terms of a full helix–coil transition, this would represent a shift difference of about 3 p.p.m. This is close to the values obtained for the Glu–Lys–Ala copolymer.

The sum total of the results to date therefore suggests a helix–coil shift difference $\Delta_{H/C}$ for the α carbon and amide carbonyl of about 3 p.p.m. that is largely independent of solvent. This is in marked contrast to the α*CH* proton spectrum for which there is considerable solvent dependence of $\Delta_{H/C}$, a parameter which is found to be the lowest of all in the solvent of greatest interest, water. These preliminary carbon spectra encourage the hope that a conformational shift difference of real diagnostic value in protein studies is to be found.

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## NOTE ADDED IN PROOF

A recent paper on poly L alanine by M. Goodman, F. Toda and N. Ueyama [*Proc. Nat. Acad. Sci., Wash.* **70**, 331 (1973)], published since this lecture was given, gives an explanation of the αC*H* 'double peaks' that accords exactly with our views: viz. the main highfield peak is a helix and coil time average, whilst the small lowfield peak is pure coil. As explained in the text, we do **not** consider this a general explanation of αC*H* double peaks, but one peculiar to poly L alanine.