NUCLEAR MAGNETIC RESONANCE AND OPTICAL STUDIES OF POLYPEPTIDE CHAIN CONFORMATION

F. A. BOVEY

Polymer Chemistry Research Department, Bell Telephone Laboratories, Murray Hill, New Jersey, U.S.A.

1. INTRODUCTION

Everyone is aware of the intense effort that has been devoted to the study of the structure of proteins and polypeptides during the last two decades, culminating in the complete x-ray determination of the structures of *myoglobin*, *hemoglobin*, *lysozyme*, and *ribonuclease*. Few problems in natural science have been subjected to such a heavy assault with such a variety of weapons. The most important of these, of course, has been x-ray diffraction. One cannot imagine at present any other technique capable of providing the thousands upon thousands of individual parameters necessary to describe a complete protein structure. In view of the spectacular triumphs of this method, why do many of us who are concerned with polypeptide and protein structure continue to employ such relatively humble techniques as optical rotation, circular dichroism, and nuclear magnetic resonance, which are capable of giving at most only a handful of structural parameters? I think there are good answers to this question, and I would suggest a few as follows:

(*a*) If our main interest is in polypeptides of a single repeating residue, then only a relatively limited number of molecular parameters may be adequate for a good description.

(*b*) X-ray studies are limited for the most part to crystalline solids and, except in certain special circumstances, cannot inform us what biopolymers are doing in solution. (One such exception is the x-ray study of polypeptides in solution by Brady, Salovey and Reddy[1] in our laboratories; this requires labelling with heavy atoms such as bromine.)

(*c*) Optical and n.m.r. methods have an intrinsic interest in their own right. This may be the most important reason for using them.

2. THE α-HELIX

This lecture will centre chiefly on the α-helix and its conformational changes, as observed by both high resolution n.m.r. and circular dichroism (CD). In particular, we shall be concerned with the α-helix-to-random coil transition. This phenomenon has, of course, been intensively studied both experimentally and theoretically, but there nevertheless are some unresolved problems. In particular, the question of why the transition occurs at all

appears to be quite unanswered for organic solvent systems. We may not be able to give a complete answer here either, but at least we will furnish some additional evidence.

3. THE HELIX–COIL TRANSITION

In *Figure 1* is shown the now very familiar structure of the α-helix. The structure represented is that of poly-L-alanine in the right-handed helical conformation. This representation claims two somewhat novel features: *first*, it is a pair of stereoscopic views; *second*, it was not drawn by a draftsman, but was generated directly on microfilm by the General Electric 645 computer, using a programme adapted for this purpose by R. L. Kornegay

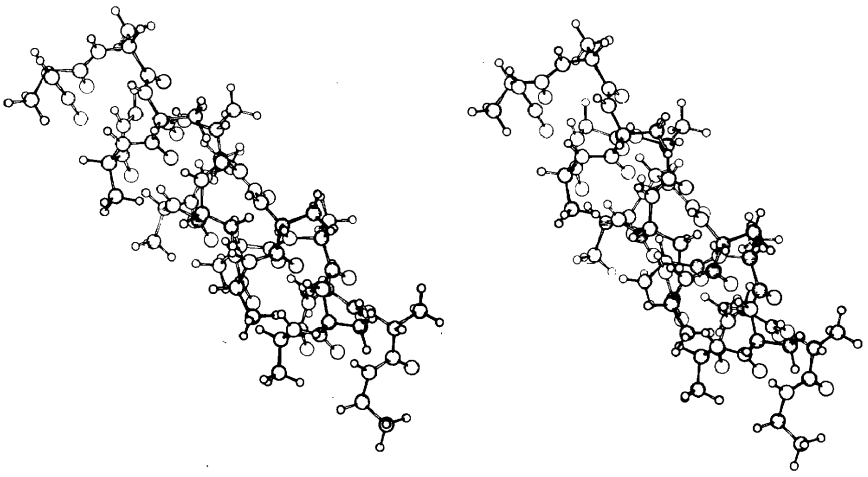


Figure 1. Stereo views of α-helix (must be viewed with a stereo lens system obtainable from Stereo-Magniscope, Inc., New York City, or from the author upon request)

(Bell Telephone Laboratories) from a more general programme devised by C. K. Johnson (Oak Ridge National Laboratories).

As it occurs in nature and in many synthetic polypeptides, the α-helix has approximately 18-fold symmetry, i.e., it repeats exactly every 18 amino-acid residues. There are 3·6 residues per turn and a residue translation of 1·49 Å, i.e., as we pass from a point in one residue to the corresponding point in the next, we move 1·49 Å along the helical axis.

In *Figure 2* are shown the absorption spectrum and the circular dichroism spectrum characteristic of a polypeptide in the α-helical conformation. These spectra are of poly-γ-methyl-L-glutamate (DP 400) in hexafluoroisopropanol solution. They are generally in agreement with those presented by Holzwarth and Doty[2], but the CD band intensities were found to be slightly smaller than they report. In *Figure 3*, the CD spectrum is decomposed into the constituent bands as now recognized, and the relative intensity of each is indicated.

The currently accepted interpretation of the circular dichroism bands, and of the corresponding bands of the absorption spectrum, is as follows:

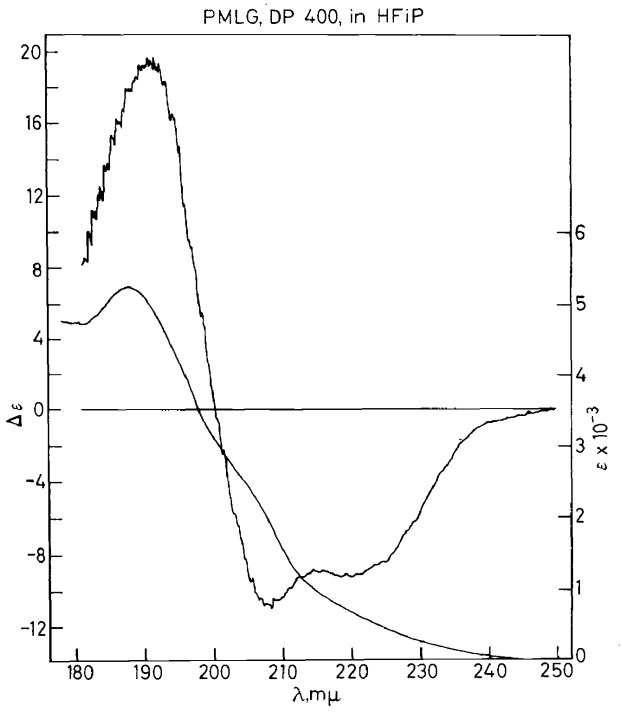


Figure 2. Absorption and CD spectra of poly-γ-benzyl-L-glutamate, 2×10^{-2} M in hexafluoroisopropanol

(*a*) At 223 mμ is the negative n–π^* peptide band, quite strong in dichroism, but weak in absorption.

(*b*) At 190 mμ and 205 mμ are the opposite-sign, exciton-split π–π^* bands of the peptide chromophor. The positive band is polarized perpendicularly to the helical axis; the negative band is polarized parallel to the helical axis. The positive band has twice the intensity of the negative band.

We are concerned also with the left-handed α-helical conformation which poly-L-aspartate chains are believed to prefer. There appear to be little if any published circular dichroism data for these polymers, the left-handed configuration being deduced from b_0 values obtained from optical rotatory dispersion measurements[4]. From our own limited data, we know that poly-β-methyl-L-aspartate and poly-β-benzyl-L-aspartate give *positive* n–π^* CD bands in about the same position as the negative n–π^* band of a right-handed α-helix.

When transition from the right-handed α-helix to the random coil occurs in aqueous solution, it has been reported[2, 3] that the negative n–π^* band gives place to a very weak positive band. (As we shall see, this is not necessarily the case in organic solvents, where a weak negative n–π^* band may persist in the random coil conformation.) The π–π^* exciton-split bands are

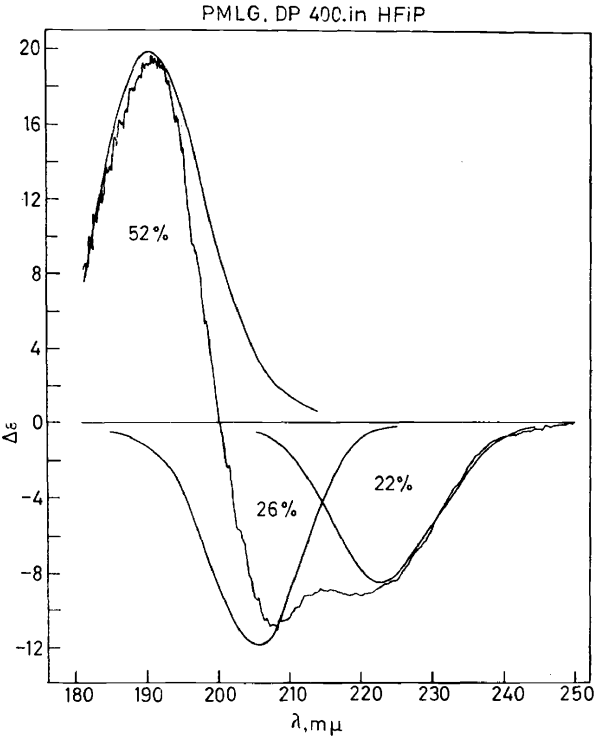


Figure 3. The CD spectrum of *Figure 2* decomposed into n–π^*, $\parallel$ π–π^*, and $\perp$ π–π^* bands

replaced by a single negative band at about 200 mμ. In the work to be described, the π–π^* transition could not be observed because of high absorption, but the n–π^* band furnished a satisfactory measure of the helix-coil transition.

4. NMR OBSERVATIONS OF THE α-HELIX–RANDOM-COIL TRANSITION

The first n.m.r. study of the helix–coil transition was reported in 1959 by Bovey and Tiers[5], who observed an extreme broadening of the n.m.r. peaks of poly-γ-benzyl-L-glutamate in trichlorethylene, but were able to see reasonably well-resolved spectra for both helix and coil on addition of appropriate quantities of trifluoroacetic acid. Several other authors have since examined this and closely related systems[6–9]. We report here a more detailed study of poly-γ-benzyl-L-glutamate (PBLG) than our previous one, and also a parallel study of poly-β-benzyl-L-asparate (PBLA). We have used chloroform as the helix-supporting solvent and trifluoroacetic acid as the helix-breaking solvent. For both studies we have used polymers of low molecular weight (DP *ca.* 50) and of high molcular weight (DP *ca.* 1000).

In *Figure 4* are shown 100 Mc/s spectra of PBLG of DP 55 in chloroform alone; with 15% TFA; and with 30% TFA. These spectra were run at 50°

and are representative of the many that have been run. All polymer solutions contained 10% (wt./vol.) of polymer. There is a marked broadening of all peaks in $CHCl_3$. The peaks of the backbone protons, NH and α-CH, are so broadened that they seem to have disappeared, as has been previously noted by Goodman and Masuda[6]. However, on increasing the spectrometer gain, they can be readily seen. The protons of the side-chain are progressively less broadened as we move out from the glutamyl methylenes (β-CH_2 at *ca.*

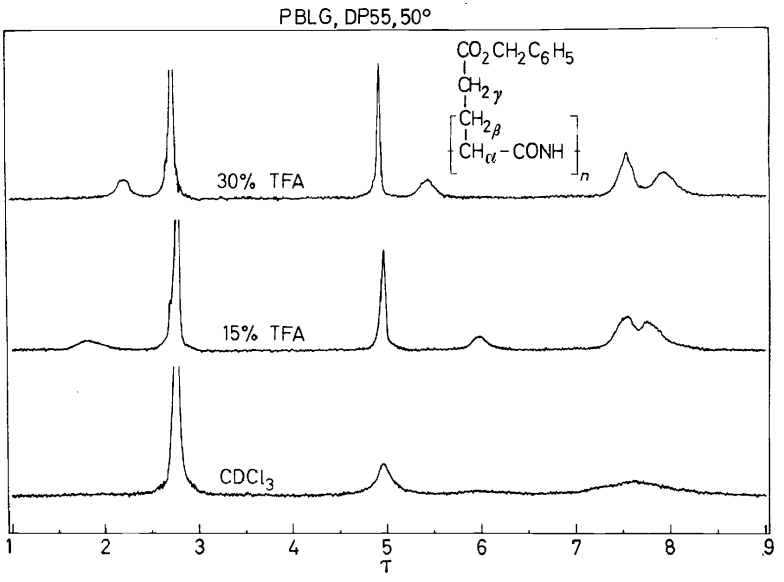


Figure 4. 100 Mc/s spectra of PBLG (10%; DP 55) in deuterochloroform alone; in 15% TFA in deuterochloroform; and in 30% TFA in deuterochloroform

7·7τ and γ-CH_2 at *ca.* 7·5τ) to the benzylic methylene group (4·96τ) and to the phenyl group (2·76τ). Upon addition of 15% of trifluoroacetic acid (as little as 2·5% has a nearly equal effect), all peaks narrow considerably, and the NH and α-CH resonances become clearly visible. However, their positions are the same and the chains must be still largely helical, for the circular dichroism measurements, which we shall discuss below, show an n–π^* band at 224 mμ with an intensity, $\Delta\epsilon$, of −6·4 l.-mole^{-1}-cm^{-1}. At 30% TFA, all peaks narrow further, but the effect is not striking. Circular dichroism measurements show that the polymer is now a random coil, the n–π^* band having nearly disappeared.

In *Figure 5* are shown corresponding spectra for the PBLG of DP 1000. In pure chloroform, the entire spectrum appears to have vanished completely when recorded over the usual 1000 c/s width. The smaller inset spectrum, recorded over a 5000 c/s sweep, shows that it is actually still there, but very greatly broadened. The only visible peak is the phenyl resonance, now about 250 c/s in width. Measurable intensity is spread out over about 4000 c/s, as integration of the spectrum clearly shows. In the presence of

2·5% TFA, the peaks are likewise extremely broad. At 15% TFA the polymer is still helical, but the peaks are very much narrower (probably by a factor of at least 100); they are, however, markedly broader than for the DP 55 polymer under the same conditions. The peaks of the random coil exhibit the same width as those of the low molecular weight polymer in the random-coil state; the spectra are indistinguishable.

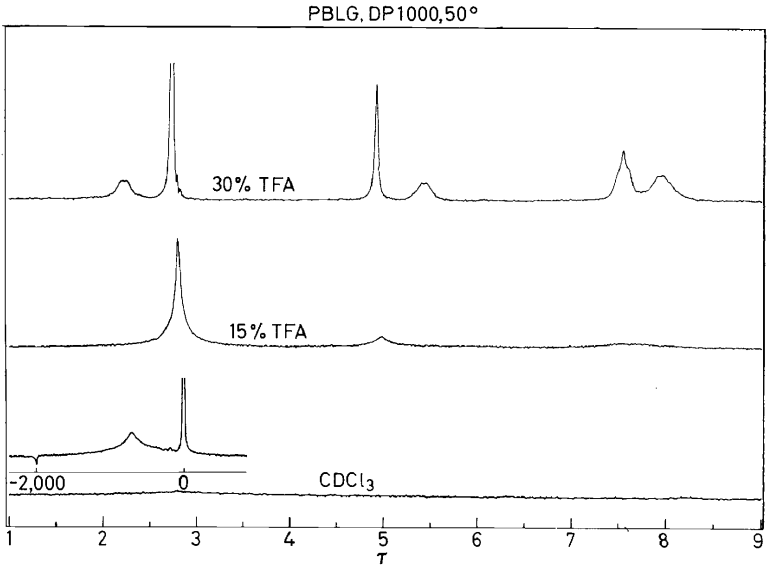


Figure 5. 100 Mc/s spectra of PBLG (10%; DP 1000) in deuterochloroform alone; in 15% TFA in deuterochloroform; and in 30% TFA in deuterochloroform

As Bovey and Tiers[5] pointed out, the extreme broadening in chloroform is very likely due to aggregation. The existence of PBLG in a liquid crystalline state under these conditions has been established[10]. The broadening is closely analogous to that exhibited by native proteins, but somewhat more extreme. It is strongly dependent upon molecular weight. Upon adding trifluoroacetic acid, these aggregates are broken up. A considerable dependence of the line-width of the free helices upon molecular weight is still noticeable, as might be expected for a rod-like macromolecule, but it is much less marked. In the random-coil state, there is no dependence upon molecular weight, since now local segmental motion determines the line-width. This last is the behaviour normally characteristic of vinyl polymers in solution.

Figures 6 and *7* show similar data for poly-β-benzyl-L-aspartate of low and high molecular weight. The spectral broadening in chloroform is comparable, but is not quite so great: both the phenyl and benzyl protons can now be discriminated. The onset of narrowing occurs at much lower acid concentration for both the low and high molecular weight polymer. In 30% TFA, the NH resonance is a doublet and the α-CH a binomial quartet, indicating approximately equal couplings (*ca.* 7 c/s) of the α-CH to the β-CH and NH protons.

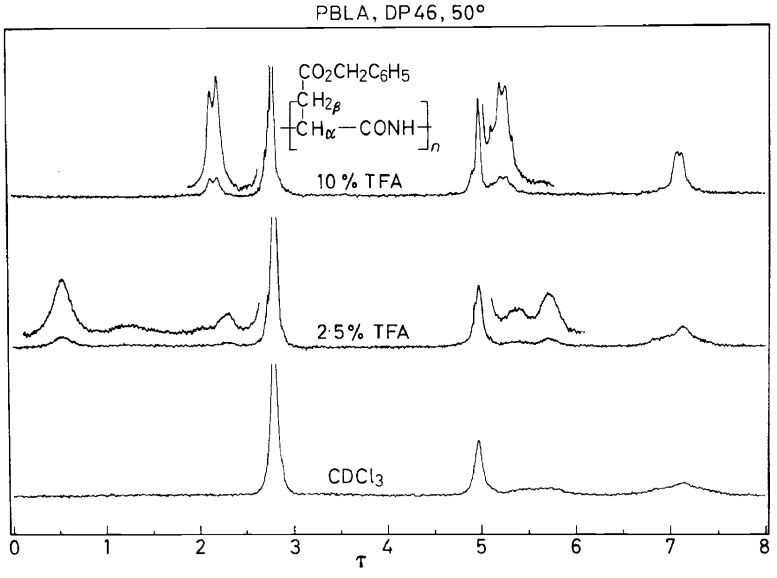


Figure 6. 100 Mc/s spectra of PBLA (10%; DP 46) in deuterochloroform alone; in 2·5% TFA in deuterochloroform; and in 10% TFA in chloroform

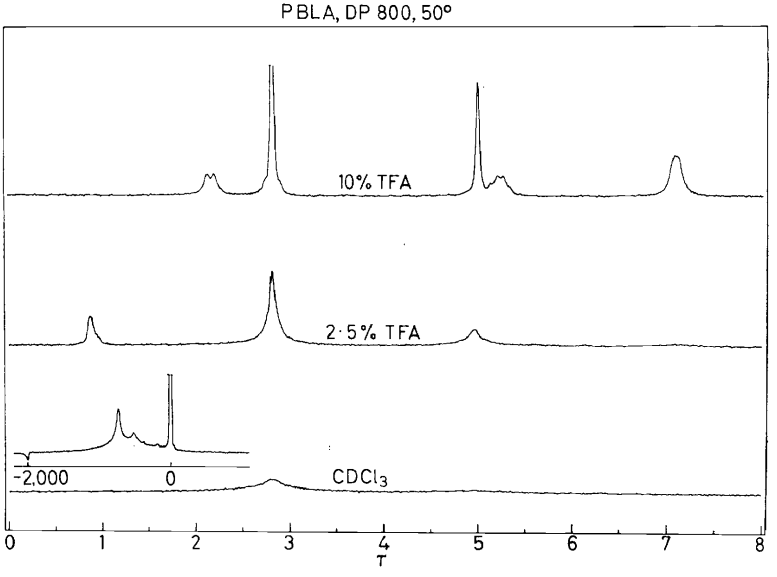


Figure 7. 100 Mc/s spectra of PBLA (10%; DP 800) in deuterochloroform alone; in 2·5% TFA in deuterochloroform; and in 10% TFA in chloroform.

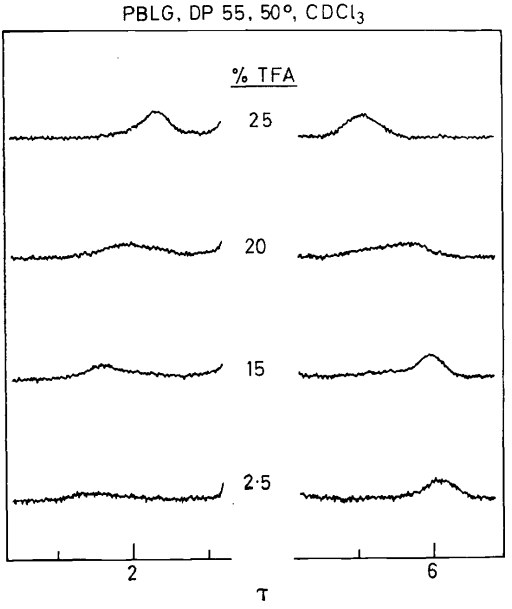


Figure 8. NH and CH peaks of PBLG (10%; DP 55) as a function of TFA conc. in $CDCl_3$.

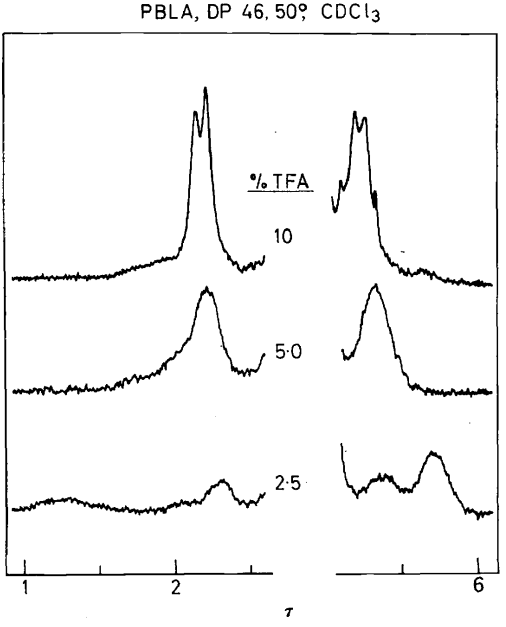


Figure 9. NH and CH peaks of PBLA (10%; DP 46) as a function of TFA conc. in $CDCl_3$

This clearly is a measure of the actual local conformation of the random coil, but unfortunately we do not at present know the dependence of the vicinal coupling upon the H—N—C—H dihedral angle.

Let us turn now to a consideration of the chemical shifts of the various polypeptide protons as a function of conformation. Certain obvious trends can be seen in *Figures 4–7*. The behaviour of the NH and α-CH protons is shown in greater detail in *Figures 8* and *9*, which represent the low-molecular-weight polymers at 50°. For PBLG, the α-CH peak remains unchanged at 6·00τ until about 20% TFA, when a down-field shift begins which is complete at 25% TFA. This corresponds to the helix–coil transition. At 20% TFA (50°), there is a clear indication of a smaller peak at 5·70τ. We believe this corresponds to those chains which are too short to sustain a helix under these conditions, and begin to undergo transition to the coil. The NH peak also undergoes a marked change in position at the transition, but it becomes *more* shielded. Similar observations have been reported by Stewart *et al.* for poly-L-alanine[8]. (*But see Note 1 added in proof on p. 432*).

These same general features are exhibited in the poly-β-benzyl-L-aspartate spectra shown in *Figure 9*. Here, however, the NH peak moves up-field more markedly when transition to the random coil occurs. We observe also that

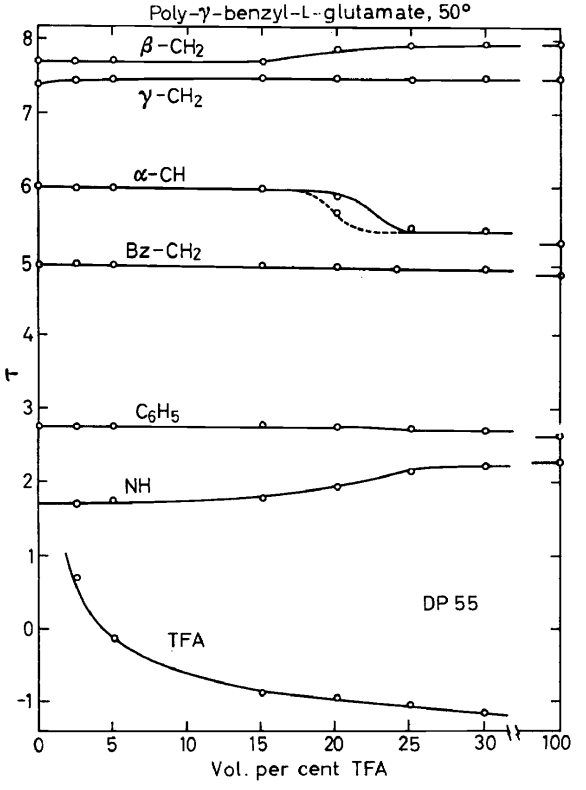


Figure 10. Chemical shifts of PBLG (10%; DP 55) plotted *versus* conc. of TFA in $CHCl_3$. Dotted curve corresponds to transition of low-molecular-weight fraction

even in chloroform alone, there is a substantial fraction of asparate chains, perhaps one-third, which presumably are too short to form a helix, and appear as a peak at *ca.* 5·3τ. Beyond the transition, i.e., at 5% or more of TFA, the helical peak disappears and this 5·3τ peak is the only one visible.

The appearance of these two α-CH peaks might at first suggest that we are about midway in the transition, and that the equilibration of magnetic nuclei between the coil and helical environments is so slow as to allow a separate peak for each to appear. This explanation raises questions however, for two reasons: (*a*) temperature jump measurements[11] indicate that the lifetimes of the helix and random-coil conformations in equilibration with each other are likely to be less than 10 microseconds—even a lifetime as long as a millisecond would still be short enough to merge the n.m r. peaks completely into a single peak, they must, therefore, correspond to non-exchanging spin populations; (*b*) the spectra of the high molecular weight PBLA (not shown here in detail) exhibit the same transition, but do not appear to show helical and coil peaks simultaneously. (*cf. Note 1 added in proof on p. 432*).

The chemical shift changes for all protons are plotted *versus* volume per-cent TFA in *Figure 10* for PBLG of DP 55. The high-molecular-weight

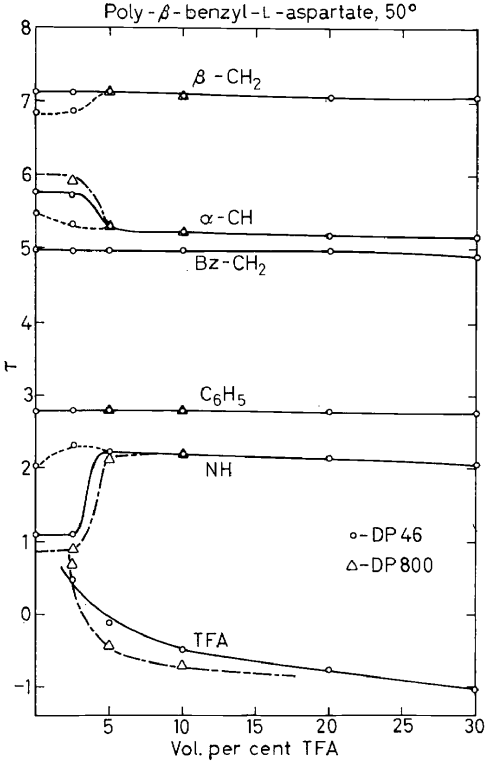


Figure 11. Chemical shifts of PBLA (10%) plotted *versus* conc. of TFA in $CHCl_3$; ○: DP 46, △: DP 800, – – – –: Low mol. wt. fraction in DP 46 polymer

polymer shows nearly the same behaviour, except that the separate transition for shorter helices (indicated by the dotted curve in the α-CH plot) does not appear. A similar plot for PBLA, DP 46, is shown in *Figure 11*. The PBLG transition between 20 and 25% TFA is clearly shown by the α-CH and NH peaks; those for side-chain protons show smaller and smaller changes the farther out they are. The β-protons show an appreciable shift in both spectra; the others exhibit small but easily observable changes.

We might at this point ask the question: how do we know that these changes in the n.m.r. spectra correspond to the helix–coil transition itself? Perhaps they are actually due to the protonation of the peptide oxygen atoms:

Such protonation is known to occur for small-molecule amides, at least if the acid is strong enough[12–14]. This might precede but not necessarily coincide with the actual transition. To answer this question, let us look a little more closely at the circular dichroism measurements. Those shown in *Figure 12* were made at 33°, using the same solutions as used for the n.m.r. measurements. Because of solvent and polymer absorption in these concentrated solutions, it was not possible to make measurements beyond

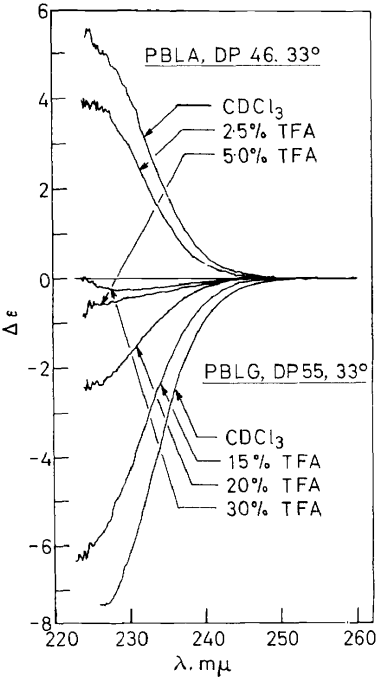


Figure 12. CD spectra of 10% solutions of PBLG (DP 55) and PBLA (DP 46) as a function of TFA conc. in $CDCl_3$

222 mμ, but this suffices to reach the n–π^* extrema for both the polyglutamate and polyaspartate systems. In *Figure 13*, the α-CH peak positions and $\Delta\epsilon$ are plotted *versus* the acid concentration. Both appear to depend on the acid concentration in the same way, and therefore one may logically (but not rigorously) conclude that both reflect the helix–coil transition.

It is well known that in organic solvent systems such as these, the helix is the form stable at elevated temperatures. If, therefore, we observe the

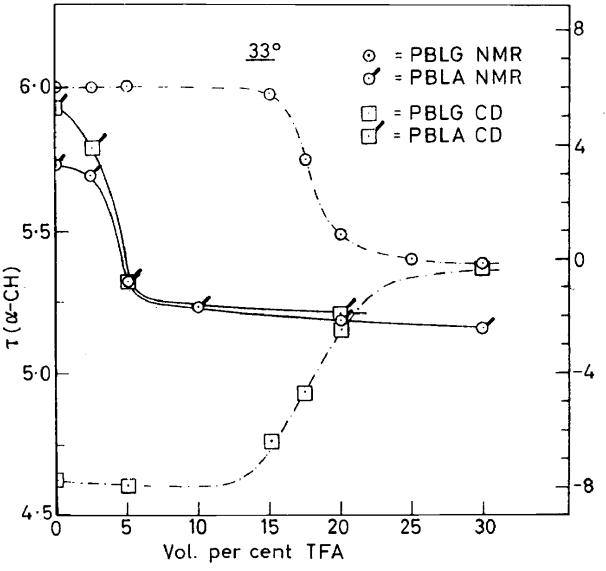


Figure 13. $\Delta\epsilon$ and $\tau_{\alpha\text{-CH}}$ of 10% solutions of PBLG (DP 55) and PBLA (DP 46) plotted *versus* TFA conc. in $CHCl_3$; 33°

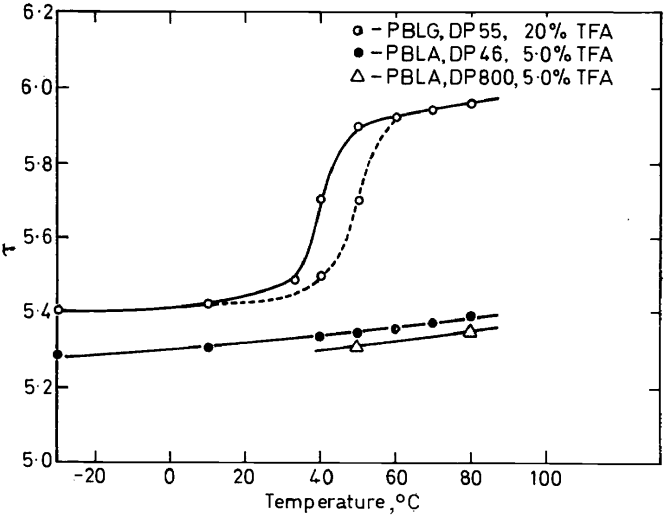


Figure 14. $\tau_{\alpha\text{-CH}}$ for PBLG (DP 55) and PBLA (DP 46 and 800) *versus* temperature

α-CH peak position as a function of temperature and if it reflects the helix–coil transition, we should see a marked up-field shift as we pass through the temperature region appropriate for the transition. This behaviour is indeed seen (*Figure 14*) for PBLG (DP 55) in 20% TFA, under which conditions the random coil is stable at *ca.* 25° and below. The transition is rather broad, as expected for a polymer of relatively low molecular weight, but seems to be complete above *ca.* 70–80°. In contrast, PBLA shows no such transition in the analogous solvent system (5% TFA). There appears to be no dependence of the aspartate helix–coil equilibrium upon temperature.

5. ORIGIN OF THE HELIX–COIL TRANSITION

These experiments raise once again a knotty and still-unanswered question: just what is it that causes the transition from helix to coil to occur in these systems as the concentration of acid is increased? The marked up-field shift of the TFA carboxyl protons caused by the polypeptide (*Figures 10* and *11*) clearly points to a strong interaction of some kind. This increased shielding has been observed for poly-L-alanine solutions by Stewart *et al.*[8] These investigators also studied the behaviour of small model molecules, *N*-methylacetamide and *N*,*N*-dimethylacetamide, in the chloroform-TFA system and found a very marked *deshielding* of the TFA protons[15]. They believed this pointed to the formation of an ion pair of protonated amide and trifluoroacetate ion. They further concluded that TFA does *not* protonate the polypeptide amide oxygens, since the change in carboxyl peak position is in the opposite direction. There is, however, infrared evidence for such polypeptide protonation. Klotz and Hanlon and their coworkers[16–18] have observed band changes in the NH overtone region which have been interpreted as indicating that protonation can occur for both model compounds and polymers, and they conclude that protonation causes the helix–coil transition. As we shall see shortly, however, there are grounds for doubting that trifluoroacetic acid is strong enough to cause protonation of either model amides or polypeptide chains.

We have found that perchloric acid is capable of causing the helix–coil transiton in poly-L-alanine (DP 25). In *Figure 15* are shown the n–π^* CD spectra for poly-L-alanine in 10^{-3} M solution in hexafluoroisopropanol, a solvent similar to trifluoroethanol and capable of supporting the α-helical conformation of this polymer. In all solvents in which we have observed it, the n–π^* band of poly-L-alanine is abnormally weak compared to other polypeptides, but we shall assume for present purposes that in hexafluoroisopropanol the polymer is nevertheless fully helical. It can be seen that the addition of only 0·003% of perchloric acid, corresponding to 0·30 molar equivalent per peptide residue, suffices to decrease the n–π^* band markedly, and that the transition to the random coil is complete when 0·9 molar equivalent is present. In contrast, the poly-L-alanine transition requires over 75 vol. percent of trifluoroacetic acid, chloroform being the other solvent[8]. Since perchloric acid is known to be very strong, at least in solvents of low basicity, surely it would seem that here, at least, protonation of the peptide units is responsible for the disruption of the helix. But once again there are grounds for doubt.

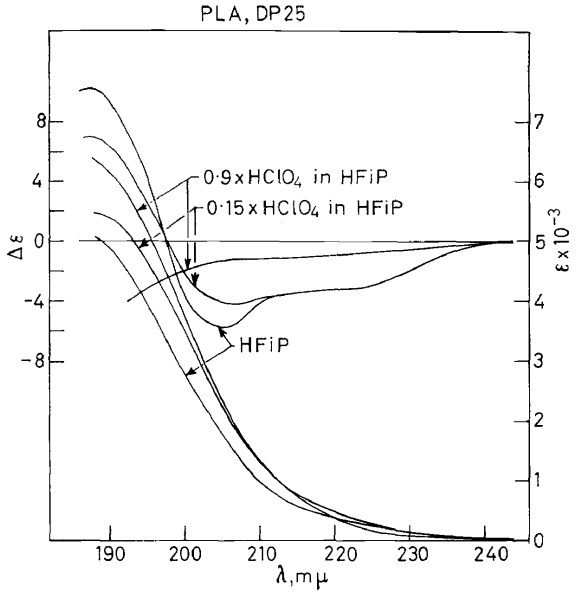
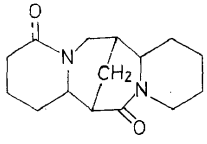


Figure 15. CD spectra of poly-L-alanine (10^{-3} M in hexafluoroisopropanol, HFiP) as a function of perchloric acid conc., expressed in moles per mole of peptide unit

The doubts have been generated by some preliminary studies of model compounds. These studies are still very incomplete, but the results appear significant. We need a model amide which has a strong n–π^* band, but which is rigid and incapable of conformational change. Small molecule amides giving observable n–π^* CD bands are very scarce; most simple amides show no observable dichroism in this region of the spectrum. Litman and Schellman[19] have observed a weak n–π^* CD band in L-3-aminopyrrolidone, and weak n–π^* bands have been found by us and by Balasubramanian and Wetlaufer[20] in a number of diketopiperazines. These latter compounds are subject, however, to possible conformational changes. A much more suitable compound is D-oxolupanine, which was observed some time ago by



Dr J. W. Longworth, then in our laboratory, to have an o.r.d. Cotton band in the n–π^* region. Its conformation appears from molecular models to be rigid. The CD spectrum of this diamide in the region of major interest is shown in *Figure 16* in a number of solvents. In chloroform, a band is seen at 230 mμ, comparable in intensity ($\Delta\epsilon = 5{\cdot}4$) to that of poly-$\beta$-benzyl-L-aspartate and remarkably strong for a nonpolymeric molecule. The CD spectrum in trifluoroethanol is similar, and even somewhat stronger

($\Delta\epsilon = 7{\cdot}2$), but shifted to 221 m$\mu$. In addition to the positive band, there is a negative band (not shown) at 202 mμ, in the same position as the absorption maximum. It seems reasonable to assign these to n–π^* and π–π^* transitions, respectively, just as in the α-helix spectrum. If protonation were to occur to a substantial extent, the n–π^* band should decrease in intensity or disappear, for if the representation of the protonated form given above is correct, the nonbonded electrons of the unprotonated carbonyl oxygen are now bonded.

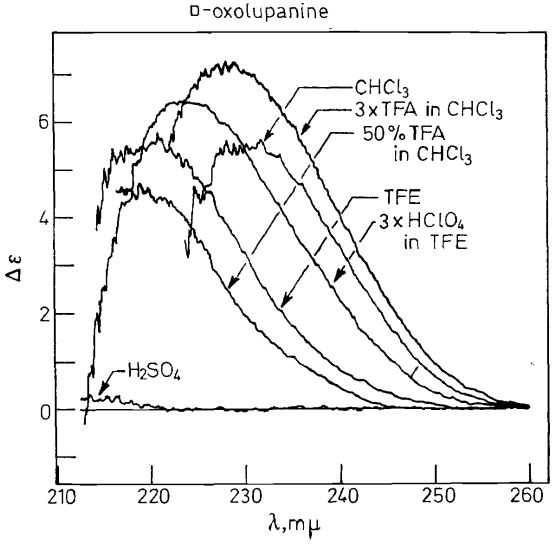


Figure 16. The n–π^* region of the CD spectrum of D-oxolupanine in $CHCl_3$ and in $CHCl_3$ with added TFA; in TFE and in TFE with added $HClO_4$; and in conc. H_2SO_4

In concentrated sulphuric acid, this band does indeed disappear. (The π–π^* band and the absorption maximum shift in position, and the former changes sign.) But in the presence of a three-fold molar excess (based on two amide residues per molecule) of perchloric acid in trifluoroethanol (TFE) and in the presence of a 3-fold and 600-fold molar excess of trifluoroacetic acid, the band does *not* disappear and even increases slightly in intensity. It appears that the amide groups in oxolupanine should be at least as strongly basic as those of a polypeptide chain, and probably more so, since they are *N,N*-dialkyl amide groups, which are normally stronger than *N*-monoalkyl amides. The carbonyl groups do not appear to be any more sterically hindered in the model than in the polypeptide.

We therefore conclude that the helix–coil transitions reported so far for nonaqueous solvents do *not* involve proton loss and gain by the polymer chain, and that other interpretations must be sought for those experimental results which have been interpreted in this way. We thus must appeal in part to the familiar hydrogen bond competition as being responsible for the transition:

$$HA\cdots HA + {-}C{=}O\cdots NH \rightleftarrows {-}C{=}O\cdots HA + HA\cdots NH$$

We assume that this equilibrium runs to the right when HA is reasonably strong, and that increasing temperature pushes it to the left for the poly-γ-benzyl glutamate system, and probably for most right-handed α-helices. It further appears that for the poly-β-benzyl-L-aspartate system, the position of this equilibrium is unaffected by temperature.

Recent potential energy calculations by a number of authors have shown that α-helical conformations tend to be preferred even in the *absence* of intramolecular hydrogen bonding, as a result of van der Waals interactions and peptide dipole–dipole interactions. Therefore, the disruption of hydrogen bonding is itself not really a sufficient answer. It must be that there is in addition a substantial difference in solvation energy between the helix and the coil aside from that involving hydrogen bonding, and that more refined energy calculations should take this into account. Also, we must of course recall that, other things being equal, there is a substantial positive entropy term which encourages random coil formation whatever the heat terms involved may be.

NMR studies of polypeptide and other biopolymer systems are being pursued in this laboratory, and elsewhere, for it appears that this approach is an effective one, particularly when combined with optical measurements.

Acknowledgement

The author acknowledges with pleasure the experimental work performed by his collaborator, Mr F. P. Hood.

Notes added in proof

1. Recently Ferretti [*Chem. Commun.* 1030 (1967)] has observed NH and α-CH peak doubling in the helix-coil transition of poly-β-methyl-L-aspartate and poly-L-leucine.) Work now in progress at 220 Mc/s in our laboratory seems to show similar behaviour for DP 1000 PBLG.

2. After the completion of this work, CD results very similar to those shown in *Figure 13* were independently reported by F. Quadrifoglio and D. W. Urry [*J. Phys. Chem.* **71**, 2364 1967)].

References

[1] G. W. Brady, R. Salovey, and J. M. Reddy. *Biopolymers* **3**, 573 (1965).
[2] G. Holzwarth and P. Doty. *J. Amer. Chem. Soc.* **87**, 218 (1965).
[3] J. P. Carver, E. Schechter, and E. R. Blout. *J. Amer. Chem. Soc.* **88**, 2550, 2562 (1966).
[4] R. H. Karlson, K. S. Norland, G. D. Fasman, and E. R. Blout. *J. Amer. Chem. Soc.* **82**, 2268 (1960).
[5] F. A. Bovey, G. V. D. Tiers, and G. Filipovich. *J. Polymer Sci.* **38**, 73 (1959).
[6] M. Goodman and Y. Masuda. *Biopolymers* **2**, 107 (1964).
[7] D. I. Marlborough, K. G. Orrell, and H. N. Rydon. *Chem. Comm.* 518 (1965).
[8] W. E. Stewart, L. Mandelkern, and R. E. Glick. *Biochemistry* **6**, 143 (1967).
[9] K.-J. Liu, J. S. Lignowski and R. Ullman. *Biopolymers*, **5**, 375 (1967).
[10] C. Robinson, J. C. Ward, and R. B. Beevers. *Disc. Faraday Soc.* **25**, 29 (1958).
[11] R. Lumry, R. Legare, and W. G. Miller. *Biopolymers* **2**, 489 (1964).
[12] G. Fraenkel and C. Niemann. *Proc. Nat. Acad. Sci.* **44**, 688 (1958).
[13] G. Fraenkel and C. Franconi. *J. Amer. Chem. Soc.* **81**, 62 (1959).
[14] R. J. Gillespie and T. Burchall. *Can. J. Chem.* **41**, 148 (1963).
[15] W. E. Stewart, L. Mandelkern, and R. E. Glick. *Biochemistry* **6**, 150 (1967).
[16] S. Hanlon, S. F. Russo, and I. M. Klotz. *J. Amer. Chem. Soc.* **85**, 2024 (1963).
[17] I. M. Klotz, S. F. Russo, S. Hanlon, and M. A. Stake. *J. Amer. Chem. Soc.* **86**, 4774 (1964).
[18] S. Hanlon. *Biochemistry* **5**, 2049 (1966).
[19] B. J. Litman and J. A. Schellman. *J. Phys. Chem.* **69**, 978 (1965).
[20] D. Balasubramanian and D. B. Wetlaufer. *J. Amer. Chem. Soc.* **88**, 3449 (1966).