Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 116-125

C-13 NMR Spectral Studies of the Thyroid Hormone Transport Protein, Transthyretin and the Pancreatic Insulin Storage Moiety, the Zinc-Insulin Hexamer

D. J. Craik, J. G. Hall, and K. A. Higgins

Victorian College of Pharmacy, Parkville, Victoria, Australia 3052

Received September 12, 1986

SUMMARY. The DEPT spectral editing technique was used to separate the CH, CH_2 and CH_3 resonances in the C-13 NMR spectra of transthyretin and the porcine zinc insulin hexamer. The advantages of this technique in terms of spectral simplification and sensitivity enhancement for ^{13}C NMR of proteins is discussed. Spin-lattice relaxation time and nuclear Overhauser effect measurements of the backbone C-α and aliphatic sidechain carbons provided information about the dynamics of the proteins in solution and the relative mobility of some sidechain groups.

INTRODUCTION. Nuclear magnetic resonance spectroscopy (NMR) provides a very powerful experimental basis for investigating the structure and dynamics of proteins in solution [1]. ^{13}C NMR spectroscopy in particular has the potential to provide information not only about molecular conformation, but also about the dynamic behaviour of specific residues within a biomolecule [2]. Two difficulties which have limited the application of ^{13}C NMR to proteins in the past are the low inherent sensitivity and the high degree of spectral overlap. There are a number of factors which contribute to the sensitivity problem in ^{13}C NMR, but one that relates specifically to proteins is the generally reduced nuclear Overhauser enhancement (NOE) observed for slow-tumbling macromolecules. The overlap problem arises simply because of the large number of inequivalent carbons in macromolecules. Both difficulties should potentially be alleviated by spectral editing techniques [3] involving polarization transfer for sensitivity gains. Such pulse techniques are

Abbreviations:

NMR, Nuclear Magnetic Resonance; DEPT, Distortionless Enhancement by Polarization Transfer; TTR, transthyretin; ZnIn, zinc insulin; NOE, Nuclear Overhauser Effect.

0006-291X/87 $1.50

beginning to be widely applied in ^{13}C NMR of small molecules for assignment purposes, but have not yet been applied to proteins. In this report we demonstrate this application in two proteins currently under investigation in our laboratories.

The thyroxine-binding protein, transthyretin (TTR), is a tetrameric protein of moderate size (M_r 54,800) whose principal function is the transport of the thyroid hormones in vertebrate blood [4]. It has also been implicated as a transport agent of the thyroid hormone, thyroxine (T4), across the blood-brain barrier [5]. The zinc insulin hexamer (ZnIn) is a smaller protein (M_r 36,000) and has been proposed as the storage form of insulin within the secretory granules of the pancreas [6]. The crystal structure and conformation of both TTR and porcine ZnIn have been defined by X-ray studies [7,8].

One specific aim in the study of these proteins is the determination of the relative flexibility of different parts of the three-dimensional structure in solution. For this purpose ^{13}C NMR spin-lattice relaxation times (T_1's) and nuclear Overhauser effects (NOE's) have been measured. Correlation times for molecular motion were then derived using the program MOLDYN [9]. We are particularly interested in mobility of serine residues in both proteins, and were therefore interested in discriminating peaks due to these residues from those due to CH backbone carbons which occur in the same spectral region. While in principle T_1 measurements should aid in this assignment process for the two types of resonance by virtue of the different number of attached protons, in practice it is shown that protein size (which affects correlation time) and the degree of internal motion can make discrimination based on T_1 difficult. This reaffirms the need for an alternative assignment tool, such as spectral editing, in the interpretation of ^{13}C NMR spectra of proteins.

MATERIALS AND METHODS. Transthyretin was obtained from human plasma using the method of Dwulet and Benson [10]. Zinc insulin used was supplied by the Commonwealth Serum Laboratories. NMR solutions of TTR were prepared by dissolving TTR (190 mg) in 5 ml of 0.2 mM Tris in D_2O. The pD of the solution was then adjusted to 7.0 with 0.1 M DCl solution. The concentration of ZnIn NMR solutions was approximately 75 mg/ml in 10% D_2O in water. The pH of the solution was adjusted using 1 M NaOH solution.

NMR measurements: ^{13}C NMR spectra were recorded on a Bruker AM300WB spectrometer utilizing a selective ^{13}C probe tuned to 75.49 MHz and using 10 mm diameter sample tubes. Routine spectra were accumulated overnight (~100,000 scans) at ambient temperatures under the following conditions: pulse width, 7 μs (60° flip angle); sweep width, 16,000 Hz; data points, 8K; acquisition time, 0.25 s; recycle time, 1 s. Low-power, broad-band, proton irradiation (0.3 W) was used during the recovery delay and Waltz decoupling [11] (0.5 W) was used during acquisition. Spectra were processed by zero-filling to 16K data points and exponential multiplication prior to Fourier transformation.

Relaxation times, T_1's, were obtained using the fast inversion recovery method [12] with a composite 180° pulse to correct for rf inhomogeneity [13] and a 90° pulse length of 13.6 μs. T_1 values were determined by non-linear three-parameter regression analysis [14]. Nuclear Overhauser effect (NOE) measurements were obtained using the gated decoupling technique [15] with recycle times on the order of 5 T_1's. DEPT subspectra were obtained using the procedure of Doddrell et al. [3]. The delay between pulses (3.33 ms) was set equal to $^1J(CH)$ of 150 Hz, and a sweep width of 12,000 Hz was used for the TTR subspectra. The CH subspectrum was generated using an additional 90° proton 'purging' pulse according to the method of Bendall and Pegg [16].

RESULTS AND DISCUSSION. The 75 MHz ^{13}C NMR spectrum of ZnIn (Fig. 1a) contains many resolved resonances, while the slightly larger protein, TTR, has

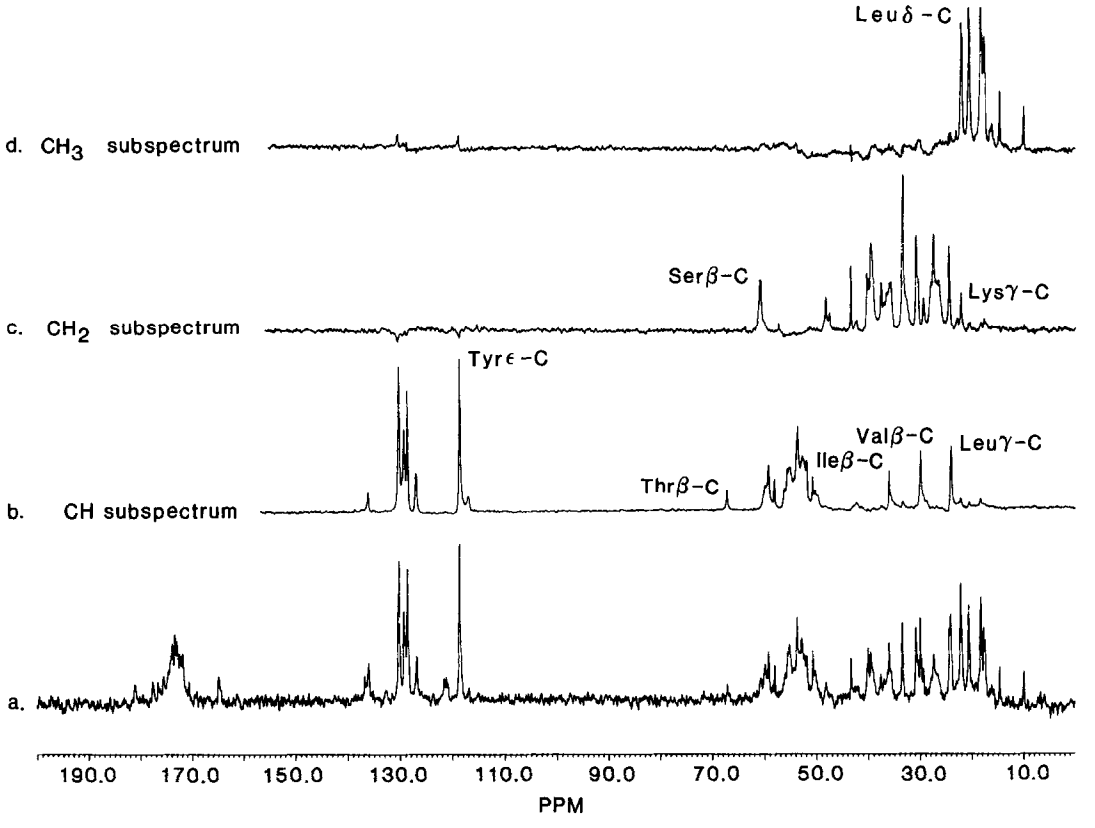

Figure 1. ^{13}C NMR Spectra of bovine ZnIn. (a) Normal Waltz decoupled spectrum, (b) CH subspectrum, (c) CH_2 subspectrum, (d) CH_3 subspectrum.

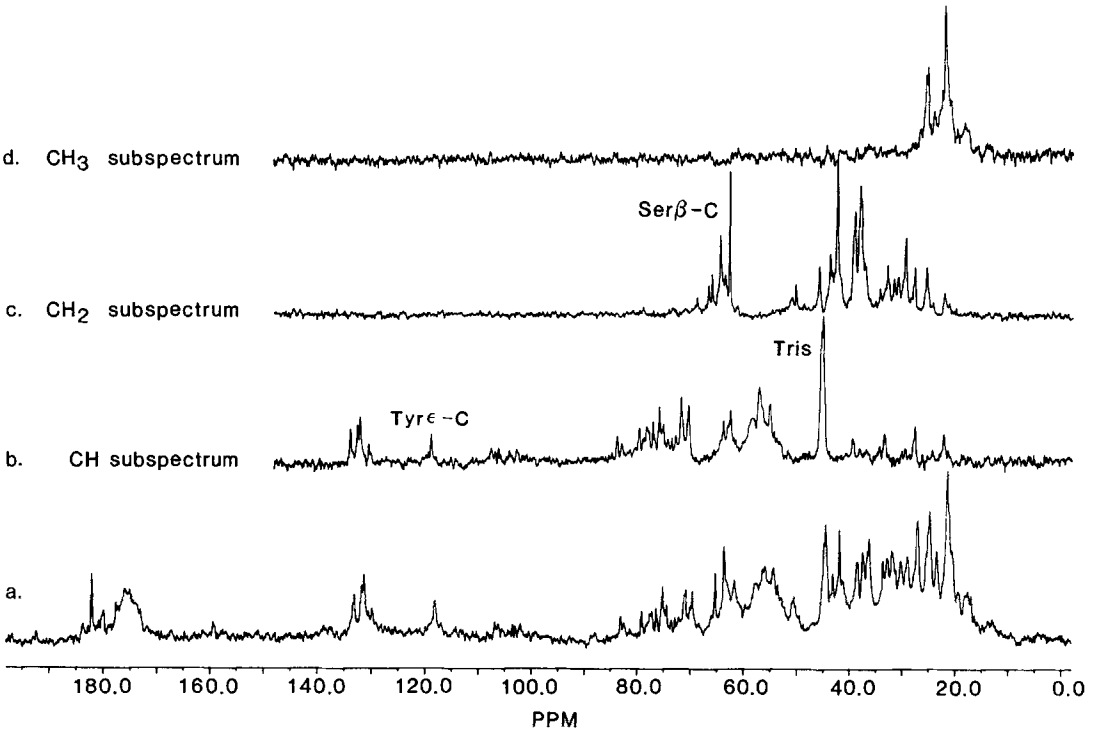

Figure 2. ^{13}C NMR Spectra of human TTR. (a) Normal Waltz decoupled spectrum, (b) CH subspectrum, (c) CH_2 subspectrum, (d) CH_3 subspectrum.

broader peaks and hence fewer resolved resonances in its ^{13}C NMR spectrum (Fig. 2a). In both cases, however, a great deal of information on solution dynamics and interactions of these proteins with ligands can be gained once peaks in the ^{13}C spectra are assigned to specific molecular sites. A number of assignment techniques have been applied to protein spectra in the past, including consideration of shifts of constituent amino acids, pH and temperature variation, studies of homologous proteins, and chemical modification. In the current study we suggest that spectral editing techniques are of particular value as a front line assignment aid in the ^{13}C NMR spectra of proteins.

The DEPT pulse sequence [3] has been used to achieve spectral editing in the current case. By varying the final proton pulse in this sequence it is possible to generate three ^{13}C subspectra containing only carbons of multiplicity CH, CH_2 or CH_3. The subspectra generated in this way (shown in Figures 1b-d for ZnIn and 2b-d for TTR) clearly provide considerable spectral simplification and allow a number of residue types to be readily identified.

For example, the C-α carbons of the backbone appear as broad envelopes in the 50-60 ppm region of the C-H subspectra (Fig. 1b and 2b), and the threonine C-β carbons appear as a distinct resonance in the CH spectrum of In and as several resolved lines between 65 and 80 ppm in the ^{13}C NMR spectrum of TTR. The serine C-β carbons are clearly resolved in the CH_2 spectra around 63 ppm (Fig. 1c and 2c). Similarly, in the CH subspectra the sidechain methine C-β carbons of isoleucine and valine, and the C-γ resonances of leucine are resolved from a large number of other overlapping peaks in the normal ^{13}C spectrum.

Many protein methyl resonances in ^{13}C NMR occur to high field of the CH_2 resonances. However, there are some overlaps, as illustrated by the CH_3 subspectrum of insulin in Figure 1d. A comparison with the CH_2 subspectrum (Figure 1c) shows that the γ-CH_2 resonance of the single lysine in insulin, which is obscured by the larger methyl signal from the six leucines present, can be visualized using DEPT.

These examples illustrate the utility of the spectral editing aspect of the DEPT technique in the interpretation of protein NMR spectra. Perhaps an even more important facet of the DEPT procedure is its sensitivity enhancement. In theory, C-H polarization transfer techniques such as DEPT should produce an enhancement of γ_H/γ_C (≈ 4) over a ^{13}C spectrum obtained without NOE enhancement. This compares with a factor-of-three enhancement produced in a conventional broadband decoupled ^{13}C spectrum relative to a spectrum without NOE enhancement. For organic molecules the theoretical sensitivity enhancement of the DEPT technique is often not realized in practice because of pulse imperfections and/or variations in C-H coupling constants within a compound. Hence the enhancement produced by DEPT is often not significantly different from that obtained in a conventional broadband decoupled ^{13}C spectrum. In the case of proteins, however, NOE enhancements are often significantly less than the theoretical maximum factor of 3, and hence DEPT has a clear sensitivity advantage.

This sensitivity advantage is illustrated in Figure 3, which shows a partial ^{13}C spectrum of insulin using broadband ^{1}H irradiation and DEPT. Note

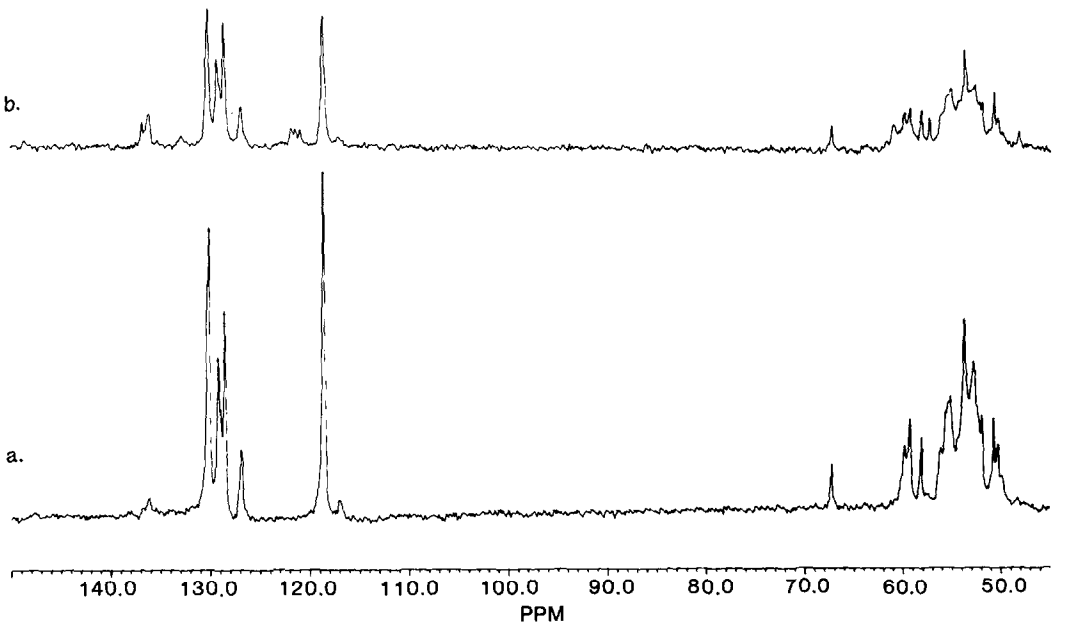

Figure 3. Illustration of signal-to-noise improvement DEPT *vs* NOE. Expansion of the backbone and aromatic region in the ^{13}C NMR spectrum of ZnIn. (a) DEPT spectrum, (b) NOE spectrum.

than even though many resonances in the upper trace show a moderate NOE (Table 1), the sensitivity gain of DEPT is substantial. The effects are even more significant in larger proteins (eg, TTR), where NOE's are often reduced to around the theoretical minimum of 1.16.

^{13}C spin-lattice relaxation time, T_1, measurements have been used extensively as a method of estimating the effective rotational correlation time (τ_R) for overall reorientation of large molecules in solution, and to identify regions of rapid internal motion within a protein [17]. The overall correlation time, τ_R, is normally obtained from T_1 data for CH backbone carbons, peaks for which usually appear in the range 50-60 ppm. However, arbitrary selection of a peak in this region may lead to a mis-estimate of τ_R, since resonances for more mobile serine sidechain CH_2 carbons can also occur in this region. As noted above, the DEPT technique overcomes this difficulty, and by aiding the unambiguous assignment of ^{13}C resonances for a number of residues, allows mobility in several regions of the proteins to be determined.

Table 1

Some ^{13}C spin-lattice (T_1) relaxation times[a,b] nuclear Overhauser (NOE) measurements, rotational correlation times (τ_R), and linewidths (LW) in transthyretin (TTR) and zinc insulin (ZnIn) for backbone (C-α), tyrosine (C-ϵ) and serine (C-β) carbons

	Transthyretin				Insulin			
	NT_1	NOE	τ_R	LW[d]	NT_1	NOE	τ_R	LW[d]
C-α (min)[c]	0.44 (0.45)	1.1 (1.2)	14	- (20)	0.22 (0.23)	1.5 (1.2)	6.3	- (9.4)
(max)[c]	0.89 (0.91)	1.1 (1.1)	29	- (40)	0.30 (0.30)	1.5 (1.2)	9.3	- (13.3)
C-ϵ Tyr	0.77 (0.78)	1.1 (1.1)	25	- (34)	0.19 (0.18)	1.6 (1.2)	5.0	- (8.0)
C-β, ser (min)	0.26 (0.26)	1.4 (1.2)	7.8	- (11)	0.30 (0.30)	1.6 (1.2)	9.2	- (13.0)
(max)	0.49 (0.50)	1.2 (1.2)	15.4	- (21)				

[a] The T_1 and NOE values are the average of two determinations. The accuracy of the T_1 and NOE determinations is estimated at ±20% for TTR and ±10% for ZnIn. NT_1's are given in seconds and correlation times in nanoseconds. (N is the number of attached protons.)

[b] Values in parentheses are theoretical values derived using the indicated correlation time.

[c] Minimum and maximum values refer to different peaks within the envelopes for C-α and C-β carbons.

[d] Overlap precluded accurate measurements of experimental linewidths but approximate values are 25 Hz for TTR and 8 Hz for ZnIn.

Under conditions of continuous proton irradiation, the relaxation of protonated carbons is exponential and is dominated by dipole-dipole interactions with directly bonded protons, even at 75 MHz [18]. The relaxation time is therefore defined by equation (1).

$$\frac{1}{T_1} = \frac{N\hbar^2\gamma_C^2\gamma_H^2}{10r_{CH}^6}\left[\frac{\tau_R}{1+(\omega_C-\omega_H)^2\tau_R^2} + \frac{3\tau_R}{1+\omega_C^2\tau_R^2} + \frac{6\tau_R}{1+(\omega_C+\omega_H)^2\tau_R^2}\right] \quad (1)$$

where γ_C and γ_H are the gyromagnetic ratios of ^{13}C and ^{1}H, N is the number of directly bonded hydrogens, r_{CH} is the C-H distance, ω_C and ω_H are the ^{13}C and ^{1}H resonance frequencies, and τ_R is the correlation time for isotropic rotational reorientation.

Table 1 contains representative values of the spin-lattice relaxation times, nuclear Overhauser enhancements (NOE's), and derived rotational correlation times for the C-α backbone carbons and some aromatic and aliphatic sidechain carbons of both TTR and ZnIn. There are two possible solutions from equation (1) for τ_R at this field-strength, and it is possible to discriminate between these by measurement of the NOE for the backbone carbons. For both proteins, the C-α carbons display a small nuclear Overhauser enhancement (1.1-1.6) consistent with a correlation time in the spin diffusion' limit

($\omega_0\tau_R > 1$). The alternative solution ($\omega_0\tau_R << 1$) would be expected to yield the maximum NOE of 2.98 and the spectra would also be expected to display far sharper resonances of less than 2 Hz linewidth, which is not consistent with the observed spectra.

Resonances within the C-H backbone region for TTR display T_1 values which range from 0.44 to 0.89 s. Analysis of these data by the MOLDYN program [9], assuming purely isotropic motion, yielded values for the rotational correlation time ranging between 14 and 29 ns. The backbone resonances for ZnIn relax slightly faster and have T_1's ranging between 0.22 and 0.31 s. The derived values of τ_R are therefore between 6.3 and 9.3 ns. It is interesting to note that substitution of these correlation times into the theoretical expression for NOE, which is related to equation (1), yields predicted NOE's in agreement with the experimental result for TTR, but not for insulin (Table 1). This suggests that the model of isotropic motion of the backbone may not be appropriate for insulin, and there may be significant internal motion in this protein relative to TTR. Multiple-field ^{13}C relaxation measurements are underway to try to better define the solution dynamics of insulin.

The serine residues in the two proteins are of particular interest to us because in both cases, some of the serines are located near potential ligand-binding sites. A number of the peaks from the 11 serine residues in TTR are resolved, and the relaxation data in Table 1 indicate a significant variation in the mobility of these residues. By contrast, the three serines in insulin yield a single peak with a relaxation time not significantly different from that of the backbone. The relative restriction of the serine residues in insulin is consistent with the fact that all three serines are located within two residues of cysteine disulphide linkages. In TTR no such restrictive linkages exist.

The serine T_1 data also conveniently illustrate the point that T_1 measurements alone cannot be used as an assignment criterion in ^{13}C NMR spectroscopy of proteins. For organic compounds, T_1 for a CH_2 group is often half that of a CH group undergoing similar motion, and so peak multiplicity

can often be decided on the basis of T_1. This also applies to TTR, where the relative mobility of the serine CH_2 groups leads to a reduction in their T_1's relative to backbone CH carbons, and the presence of two protons leads to a further reduction in T_1. The result is that in TTR serine T_1's (0.13-0.25 s) are quite distinct from backbone CH's (0.44-0.89 s) which occur in the same chemical shift region. By contrast, insulin is a slightly faster-tumbling protein, and has a correlation time closer to the T_1 minimum described by equation (1), with the result that variations in internal motion (eg of serines) have a smaller effect on observed T_1 values. The net result is a reduced discrimination between serine T_1 values (0.15 s) and those of backbone CH carbons in the same spectral region (0.22-0.30).

In summary, it has been shown that the DEPT technique is extremely valuable for the simplification and assignment of protein ^{13}C NMR spectra, and that significant gains in sensitivity can be achieved relative to conventional broadband decoupled spectra. The technique has been applied to proteins of 36,000 (ZnIn) and 54,000 (TTR) molecular weight. Subsequent to assignments being made with the aid of DEPT, relaxation time measurements have provided information on the mobility of these proteins in solution.

ACKNOWLEDGEMENTS. This work was supported by the National Research Fellowships Scheme and in part by the National Health & Medical Research Council. We thank Peter Andrews for helpful discussions.

REFERENCES

1. (a) Campbell, I.D. and Dobson, C.M. (1979) Methods Biochem. Anal., 25, 1-133; (b) Jardetzky, O. and Roberts, G.C.K. (1981) NMR in Molecular Biology, Academic Press, New York; (c) Wagner, G. (1983) Q. Rev. Biophys., 16, 1-57.
2. Howarth, D.N. and Lilley, D.M.J. (1978) Prog. NMR Spectrosc., 12, 1-40.
3. (a) Doddrell, D.M., Pegg, D.T. and Bendall, M.R. (1982) J. Magn. Reson., 48, 323-327; (b) Pegg, D.T., Doddrell, D.M. and Bendall, M.R. (1982) J. Chem. Phys., 77 2745-2752.
4. Larsson, M., Pettersson, T. and Carlstrom, A. (1985) Gen. Comp. Endocrinol., 58, 360-375.
5. Dickson, P.N., Howlett, G.J. and Schreiber, G. (1985) J. Biol. Chem., 260, 8214-8219.
6. Blundell, T., Dodson, G., Hodgkin, D. and Mercola, D. (1972) Adv. Protein Chem., 26, 279-402.
7. Blake, C.C.F., Geisow, M.J., Swan, I.D.A., Rerat, C. and Rerat, B. (1974) J. Mol. Biol., 88, 1-12.

8. Blake, C.C.F., Geisow, M.J., Oatley, S.J., Rerat, B. and Rerat, C. (1978) J. Mol. Biol., 121, 339-356.
9. Craik, D.J., Kumar, A. and Levy, G.C. (1983) J. Chem. Inf. Comp. Sci., 23, 30-38.
10. (a) Dwulet, F.E. and Benson, M.D. (1983) Biochem. Biophys. Res. Commun., 114, 657-662; (b) Dwulet, F.E. and Benson, M.D (1984) Proc. Natl. Acad. Sci. USA, 81, 694-698.
11. (a) Shaka, A.J., Keeler, J., Frenkiel, T.A. and Freeman, R. (1983) J. Magn. Reson., 52, 335-358; (b) Shaka, A.J., Keeler, J. and Freeman, R. (1983) J. Magn. Reson., 53, 313-340.
12. Canet, D., Levy, G.C. and Peat, I.R. (1975) J. Magn. Reson., 18, 199-204.
13. (a) Levitt, M.H. and Freeman, R. (1979) J. Magn. Reson., 33, 473-476; (1982) 50, 95-110.
14. (a) Sass, M. and Ziessow, D. (1977) J. Magn. Reson., 25, 263-276; (b) Kowalewski, J., Levy, G.C., Johnson, L.F. and Palmer, L. (1977) J. Magn. Reson., 26, 533-536.
15. Levy, G.C., Lichter, R.L. and Nelson, G.L. (1980) Carbon-13 Nuclear Magnetic Resonance Spectroscopy, Wiley, New York, 2nd edn, p. 228.
16. Pegg, D.T. and Bendall, M.R. (1984) J. Magn. Reson., 60, 347-351.
17. (a) Allerhand, A., Doddrell, D.M., Glushko, V., Cochran, D.W., Wenkert, E., Lawson, P.J. and Gurd, F.R.N. (1971) J. Am. Chem. Soc., 93, 544-546; (b) Oldfield, E., Norton, R.S. and Allerhand, A. (1975) J. Biol. Chem., 250, 6368-6380.
18. Wilbur, D.J., Norton, R.S., Clouse, A.O., Addleman, R. and Allerhand, A. (1976) J. Am. Chem. Soc., 98, 8250-8254.