16. Valley, J. W., in *Reviews in Mineralogy*, (eds. Valley, J. W., Taylor, H. P. and O'Neil, J. R.), 1986, vol. 16, pp. 445–481.
17. Javoy, M., Pineau, F. and Delorme, H., *Chem. Geol.*, 1986, **57**, 41–62.
18. Soman, K., Unpublished PhD thesis, People's Friendship University, Moscow, 1980.

ACKNOWLEDGEMENTS. M. S. thanks the Department of Science and Technology, Government of India, for project support to study deep crustal fluid processes.

Received 21 July 1992; accepted 27 July 1992

RESEARCH COMMUNICATIONS

Thermally induced structural changes in wool: A high-resolution solid-state carbon-13 CPMAS NMR study

N. R. Jagannathan*, Ashok A. Itagi† and V. Subramaniam†

*Department of Crystallography and Biophysics, University of Madras, Guindy Campus, Madras 600 025, India
†Department of Textile Technology, A. C. College of Technology, Anna University, Madras 600 025, India

Carbon-13 CPMAS NMR spectra of merino fine wool fibre (Quality 64S) were recorded as a function of heating from room temperature to 190° C. At about 170° C, the unsaturation of aromaticity increases. Analysis of the observed chemical shift values (at 170° C) of the main chain carbonyl carbons reveal that both the right-handed α-helix and β-sheet forms exist in the wool fibre.

SOLID-STATE NMR is a powerful and versatile tool for the study of structure, morphology and dynamics of biopolymeric systems. It is well documented in the literature that the carbon-13 chemical shifts of biological molecules in the solid state observed through the cross-polarization and magic angle spinning (CPMAS) techniques are conformation dependent[1]. In particular, the C-13 chemical shifts in polypeptides and other systems arise mainly from the local conformation of the amino-acid residues and are not strongly influenced by the specific amino-acid sequences. The chemical shifts are therefore interpretable in terms of secondary structural features such as α-helix, β-sheet, ω-helix and 3_1-helix[1].

C-13 CPMAS NMR studies of silk[2–5], collagen[6], elastin[7] and tropomyosin[8] have demonstrated that this technique is very useful for obtaining information about the secondary structure of these molecules. On the other hand, studies relating to wool are meagre[1,9] and we report here the results of a study of the conformational response to thermal heating of the merino fine wool (Quality 64S).

In spite of its long use as a fibre, the structure and morphology of wool is still not understood well. Wool is relatively more complex in composition and structure than silk and it contains a large number of amino acid side chains of all types occurring in different proportions[10]. On the basis of X-ray diffraction data, native wool can be divided into two general categories: the α- and β-keratins. Chemical analyses indicate that no particular amino acid predominates but that there is a high content of polar residues, cysteine and proline in the keratins. It is in fact surprising that the keratins give such 'crystalline' X-ray fibre patterns even in the presence of these polar residues. However, the heavy cross-linking, presumably derived from S–S bridges, renders keratins in the native state difficult to characterize. The non-crystalline material acts as a highly cross-linked amorphous polymer in a rubbery state[10,11].

Much of the complexity of wool behaviour and the effects of temperature, stress and chemical reagents are due to two factors: (a) the ease of occurrence of the crystal transitions from α to β or to random coil, and (b) the ease of breakdown of cross links in amorphous regions and the formation of new ones. Moreover, X-ray diffraction study also indicates that if the heating is sufficiently small, the phase change at the molecular level is from the α-helical structure to the β-extended structure. On the other hand, if the rate of heating is sufficiently high and cooling is rapid little or no β-structure is formed. In fact, there may be recrystallization of α material. Therefore, at a low heating rate the change that takes place is α→noncrystalline →β with a small entropy and heat change. If the heating rate is sufficiently high it is from α→noncrystalline with large entropy and heat change[12]. The complexity of wool keratin structure and its behaviour with heating, prompted us to study the material by solid state C-13 CPMAS NMR with a view to understand the effects of heat on wool fibres and to arrive at some conclusion regarding the unsaturation of wool and the conformational changes.

Merino fine wool fibres (Quality 64S) were cleaned by subjecting them to petroleum ether extraction. The cleaned fibres were dried at room temperature and samples of about 1 g were heat-treated in an oven under atmospheric conditions at 120°, 150°, 170° and

*For correspondence

190° C for specified periods. These samples were then subjected to NMR investigations.

The C-13 CPMAS NMR spectra were recorded using a Bruker MSL 300 NMR spectrometer operating at a C-13 frequency of 75.47 MHz with the total side band supression pulse sequence, TOSS. The samples were packed tightly in a cylindrical aluminium oxide rotor and were spun at 3.5 kHz. A contact time of 0.5 ms was used for all the samples with a repetition time of 5 s between successive pulsing. A phase alternation pulse sequence was used to eliminate base line and other artefacts. About 10,000 scans were accumulated for each sample with a reasonably good signal-to-noise ratio. The chemical shifts were externally referenced with respect to TMS. In the dipolar dephasing experiment a delay time of 50 μs was introduced immediately after the 90° proton pulse and preceding the decoupling pulse[13]. This method provides a means of discriminating between resonances of carbon atoms which bear protons from those which do not.

Figure 1 shows the C-13 CPMAS TOSS and the dipolar dephased spectra of the merino fine wool, while Figure 2 shows the spectra of the wool samples heated at 170° C for various timings.

The C-13 CPMAS spectra of wool can be conveniently resolved into four distinct broad peaks and reflects the complex nature of the fibre. The peaks are attributable to a variety of carbon species such as carbonyl, aromatic C_α-methine and side-chain aliphatic carbons and they resonate at about 171 to 175, 120 to 140, 45 to 60, and 10 to 30 ppm respectively. The methyl resonance peaks of Ile, Val, Leu appear at about 12 to 23 ppm, especially the peak of the Ile methyl carbon clearly appears at about 16 ppm in the dipolar dephased spectrum (Figure 1*b*). The resonance in the region of 40 to 60 ppm is due to the aliphatic methine

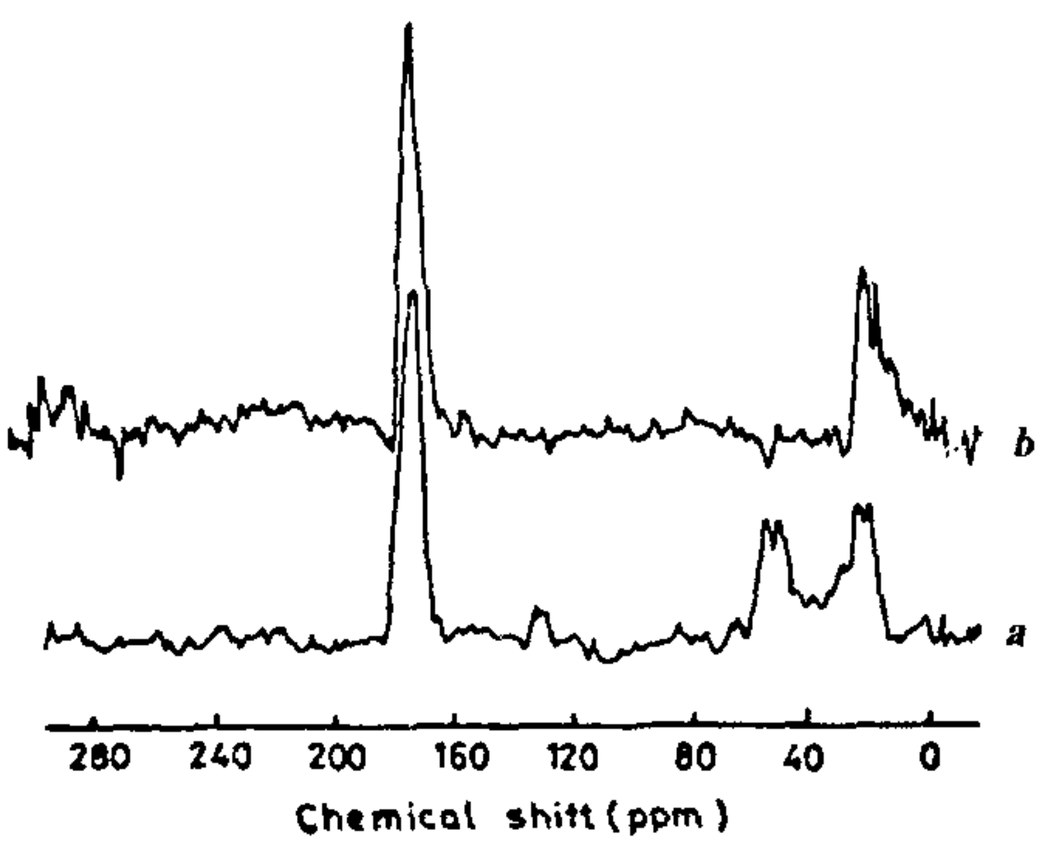

Figure 1. C-13 CPMAS TOSS NMR spectra of merino fine wool (Quality 64S). (*a*) normal and (*b*) dipolar dephased.

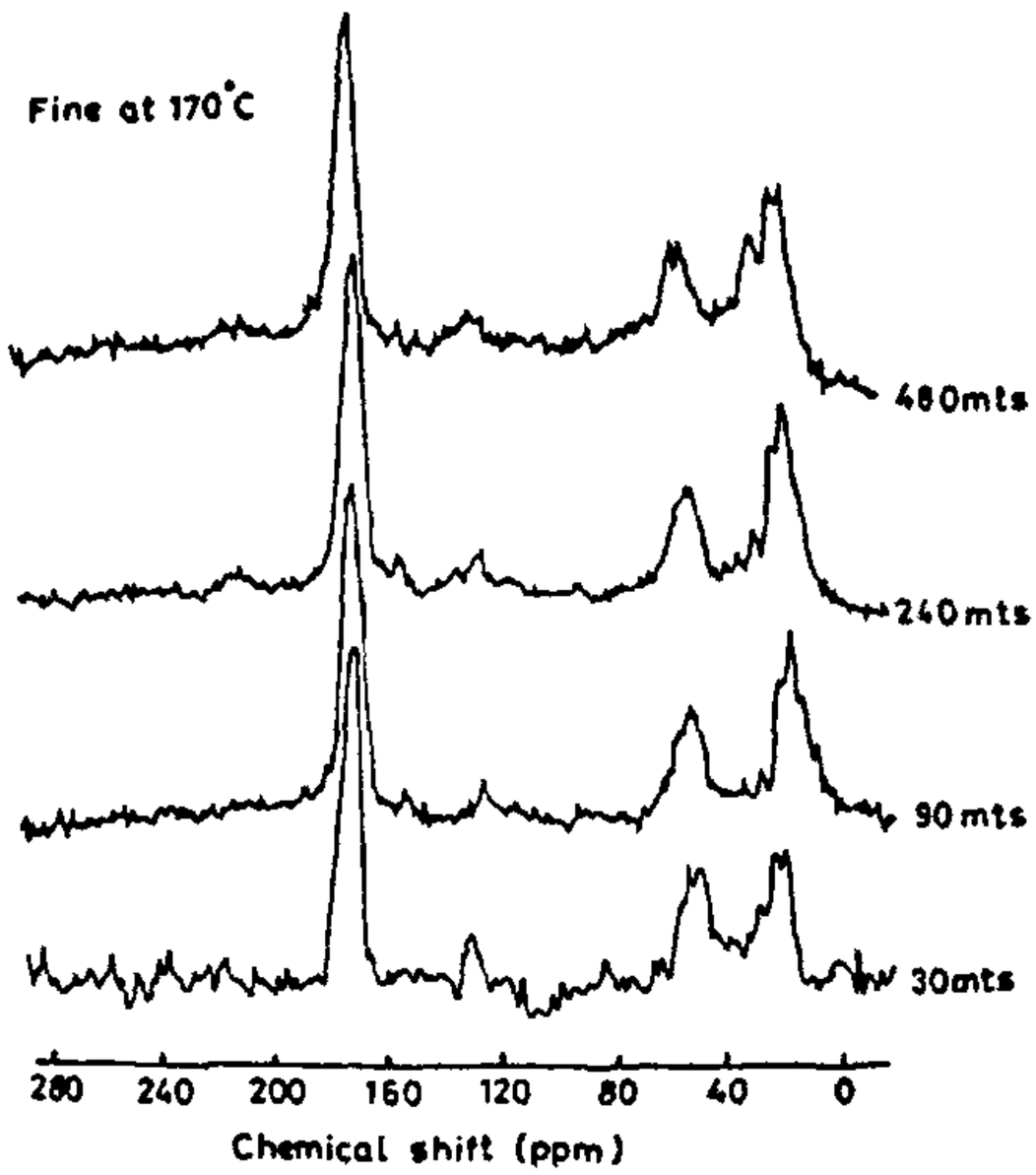

Figure 2. C-13 CPMAS TOSS NMR spectra of merino fine wool sample heated at 170° C for various timings as indicated.

and methylene carbon of the type $CH_{1,2}$-X with X = N, S, O. This is well corroborated by the disappearance of this peak in the dipolar dephased spectrum. The peak around 130 ppm is due to the aromatic carbons from the amino acids, especially the carbon species of the type C=C. Again this peak is absent in the interrupted decoupled spectrum. The carbonyl peak appears around 171 to 175 ppm[1,9].

Heating the wool from 120° to 160° C in air for various timings from 30 min to 8 h causes discoloration but without much change in the C-13 NMR spectrum. At 170° C, however, we notice some changes as shown in Figure 2. The peak at 130 ppm due to the aromatic carbons shows an increase in intensity with respect to the area of the main chain carbonyl carbon peaks which remains more or less constant during heating. This possibly indicates an increase in the C=C content of the keratin. The increased C=C content may be due to the formation of aromatic species or dehydrogenation of aliphatic carbons. Structural changes in wool fibre induced by heating could also increase mobility of the aromatic carbons. It may also be noticed that the rate of increase in the C=C content is slow in the beginning but becomes faster as the heating is prolonged as seen in the area under the curve (Table 1). The aromatic C=C contents increase by 59% compared to the decrease of the aliphatic contents by 14%. The C=O content remains fairly constant

Table 1. Details of wool sample heated at 170° C

Heating time (min)	Area* under aliphatic species@ (20–55 ppm)	Area* under the aromatic species# (130 ppm)	Area* under carbonyl species (171–179 ppm)
30	87.0	8.1	75.6
90	85.9	8.6	74.1
240	83.0	13.2	76.9
480	75.4	19.8	76.8

@ Percentage change in area under aliphatic species = 14%.
# Percentage change in area under aromatic species = 59%.
* In arbitrary units (sq. cm.)

throughout the heating; there is, however, an initial decrease, suggesting that the wool is being decarboxylated with the evolution of carbon dioxide.

Loss of gaseous volatiles[14], amide cross linking[15] and the formation of new amino acids[16,17] is reported while heating the wool above 150° C. It has also been observed that the wool begins to suffer thermal damage above 200° C (refs. 10, 18–20). IR studies of wool heated to 440° C suggest that C=C bonds are formed[21]. In our study of merino fine wool by C-13 CPMAS NMR study, it is evident that the formation of unsaturated carbons occurs at much lower temperature.

For characterization of the main-chain conformation of polypeptides and proteins in the solid state it is useful to use the C-13 chemical shift values of the main-chain carbonyl carbons because they are strongly influenced by the conformation of the main chain but not by the various amino acids and or by specific amino-acid sequences[1]. The C-13 peaks of the main-chain carbonyl carbons in the right-handed α-helix and β-sheet forms appear at 175.8 ± 0.8 and 170.9 ± 1.2 ppm respectively[22,23] In the C-13 CPMAS NMR spectra of wool samples shown in Figure 1 the major peak at 174.8 ppm is assigned to the main-chain carbonyl carbons in the α_R helix form and the other major peak at about 171.5 ppm to the β-sheet form. One minor peak at about 179.2 ppm is from the side-chain carbonyl carbons of Asp C_γ, Glu C_δ and carboxymethyl L-cysteine C_ε (refs. 1, 4). C-13 CPMAS intensities have previously been used to determine a quantitative estimate of the secondary structure in polypeptides and wools[1,24]. A similar line-shape analysis was carried out for the merino wool reported here. From the deconvolution of the three carbonyl resonances, the peak intensities were calculated and found to be constant for the whole temperature range as well as for different heating times. This possibly indicates the existence of both the right-handed α-helix and β-sheet forms in the wool fibre and ratio between them also remains constant for different heating times. The heat treatment has thus induced little conformational changes in the wool fibre.

It is interesting to note from Figure 2 that the peak near 30 ppm in the spectrum of wool grows in intensity as the heating time is increased. This resonance has been assigned to the *n*-alkyl methylene (CH_2) carbons of the lipids in the cell membrane[1]. In general, wool contains lipids of 2.5% wt in the wool cell membrane. Moreover, these methylene carbons undergo fast transition between *trans*- and gauche-isomers at room temperature as has been noticed in the case of polypeptides having a long *n*-alkyl CH_2 side-chains[25] or long alkane CH_2 side-chains[26], in which the methylene peaks appear at about 30 ppm. In the dipolar dephasing experiment we notice the disappearance of this lipid peak for the native wool. However, in the dipolar dephasing experiment carried out on the wool sample heated at 170° C for 480 min, this peak was observed clearly, probably due to long spin–spin relaxation time T_2 for this carbon compared to others. This possibly indicates an increase in mobility at longer heating times.

Thus it is clear that the C-13 CPMAS NMR appears to be a sensitive method for studying thermally induced structural changes in wool keratins. The C-13 CPMAS NMR spectra of merino fine wool recorded as a function of heating from room temperature to 190° C also revealed that the unsaturation of aromaticity increases at about 170° C. From the analysis of the C-13 chemical shifts of main-chain carbonyl carbons it is noticed that both the right-handed α-helix and β-sheet forms exist throughout the temperature range of study.

1. Yoshimizu, H. and Ando, I., *Macromolecules*, 1990, **23**, 2908–2912 and references therein.
2. Saito, H., Iwanaga, Y., Tabeta, R. and Asakura, T., *Chem. Lett.*, 1983, 427–430.
3. Saito, H., Tabeta, R., Asakura, T., Iwanaga, Y., Shoji, A., Ozaki, T. and Ando, I., *Macromolecules*, 1984, **17**, 1405–1412.
4. Asakura, T., Kuzuhara, A., Tabeta, R. and Saito, H., *Macromolecules*, 1985, **18**, 1841–1845.
5. Asakura, T., Yoshimuzu, H. and Yoshizawa, F., *Macromolecules*, 1988, **21**, 2038–2041.
6. Saito, H., Tabeta, R., Shoji, A., Ozaki, T., Audo, I. and Miyata, T., *Biopolymers*, 1984, **23**, 2279–2297.
7. Kricheldorf, H. R. and Muller, D., *Int. J. Biol. Macromolecules*, 1984, **6**, 145-151.
8. Tuzi, S. and Ando, I., *J. Mol. Struct.*, 1989, **196**, 317-325.
9. Carr, C. M. and Gevasimowicz, W. V., *Textile Res. J.*, 1988, **58**, 418-421.
10. Fraser, R. D. and MacRae, T. P., *Conformation in Fibrous Proteins*, Academic Press, New York, 1973.
11. Walton, A. G. and Blackwell, J., in *Biopolymers*, Academic Press, New York, 1973.
12. Haly, A. R. and Snaith, J. W., *Textile Res. J.*, 1970, 40, 142 146.
13. Opella, S. J. and Frey, M. H., *J. Am. Chem. Soc.*, 1979, 101, 5854 5856.
14. Launer, H. F. and Black, D., *Appl. Polym. Symp.*, 1971, **18**, 347 352.
15. Asquith, R. S. and Otterburn, M. S., *Appl. Polym. Symp*, 1971, **18**, 113 125.
16. Michlik, I., Klinger, T., Setnicka, A., Karkoska, P. and Blazej, A., *Textile Res. J.*, 1970, 40, 484 487.
17. Milligan, B., Holt, L. A. and Caldwell, J. B., *Appl. Polym. Symp.*, 1971, **18**, 113 125.

18 Menefee, E. and Yee, G., *Textile Res. J.*, 1965, **35**, 801–812.
19 Watt, I. C., *Textile Res. J.*, 1975, **45**, 728–735.
20 Marshall, R. C., Souren, I. and Zahn, H., *Textile Res. J.*, 1983, **53**, 792–794.
21 Ingham, P. E., *J. Appl. Polym. Sci.*, 1971, **15**, 3025–3041.
22 Shoji, A., Ozaki, T., Saito, H., Tabeta, R. and Ando, I. *Macromolecules*, 1984, **17**, 1472–1479.
23. Saito, H. and Ando, I., *Annu. Rep. NMR Spectrosc.*, 1989, **21**, 208.
24 Muller, D. and Kricheldorf, R. H., *Polym. Bull.*, 1981, **6**, 101–108.
25. Yamanobe, T., Tsukahara, M., Komoto, T., Watanabe, J., Ando, I., Uematsu, I., Deguchi, K., Fujito, T. and Imanari, M., *Macromolecules*, 1988, **21**, 48–50.
26. Mohanty, B., Komoto, T., Watanabe, J., Ando, I. and Shibashi, T., *Macromolecules*, 1989, **22**, 4451–4455.

ACKNOWLEDGEMENTS. The use of Bruker MSL 300 NMR spectrometer at the Sophisticated Instruments Facility, Indian Institute of Science, Bangalore, is gratefully acknowledged. N.R.J. thanks the Indian National Science Academy, New Delhi, for a Fellowship and the Department of Science and Technology, New Delhi, for financial assistance.

Received 29 June 1992, accepted 28 July 1992

Effect of 5,5-diphenylhydantoin on carotenoid pigments in human serum using laser Raman spectroscopic technique

Aranya B. Bhattacherjee, Koel Chaudhury, M. M. Bajaj and D. C. Jain*
Department of Physics and Astrophysics, University of Delhi, Delhi 110 007, India
*Department of Neurology, Safdarjung Hospital, New Delhi 110 029, India

We have applied the laser Raman spectroscopic technique to demonstrate the side effect of the anticonvulsant drug (5,5-diphenylhydantoin) on the human serum. It has been found that the drug–plasma interaction induces significant changes in the intensity ratio of β-carotenoid bands. This suggests that the plasma-bound carotenoids may be involved in drug–plasma interaction. Repetitive measurements on the serum of both normal controls as well as grand mal epileptic cases undergoing anticonvulsant drug therapy showed that, apart from the three maxima at 1535, 1162 and 1010 cm^{-1}, additional three weak bands of β-carotene were detected at 980, 1193 and 1220 cm^{-1}. These newly detected Raman bands were not seen to be affected as a result of 5,5-diphenylhydantoin drug therapy.

THE Raman spectroscopic technique is an excellent method for examining the vibrational energy levels of biomolecules, which are sensitive to the chemical nature of the constituent groups and intermolecular interactions[1]. Studies on carotenoids in blood plasma and their direct relationship to diseases using Raman spectroscopy have been carried out by Larsson and Hellgren[2]. Rein *et al.*[3] detected and identified carotenoid pigments in human blood plasma using resonance Raman technique. Extensive work on the triplet state of all-*trans*-β-carotene using Raman spectroscopy has been reported by Jensen *et al.*[4]. Recent biological and biomedical applications of Raman spectroscopy include the study of membrane-bound proteins[5] and identification of phosphate-type kidney stones.[6] Several biophysical and biochemical studies have been carried out on the plasma levels of anticonvulsant drugs which are seen to be bound to plasma proteins. Phenytoin (5,5-diphenylhydantoin), the anticonvulsant drug (Figure 1), is known to displace the normal protein-bound substances from their binding sites.[7] Manfait *et al.*[8] have applied the Raman effect to the study of drug–DNA interactions.

The present study indicates that although Raman spectroscopy of grand mal epileptic cases undergoing anticonvulsant drug therapy (5,5-diphenylhydantoin) over a prolonged period $\{(GME)_p\}$ and normal controls (NC) are similar in some respects, there are significant intensity ratio variations, which will be discussed later. During investigation of drug–plasma interactions, three additional β-carotenoid peaks were detected corresponding to 961 cm^{-1}, 1193 cm^{-1} and 1220 cm^{-1}. Thus we have succeeded in showing that the changes in the relative intensities of β-carotenoid bands in human serum studied by Raman spectroscopy provide additional insight in understanding the biochemistry of the anticonvulsant drug–plasma interaction.

Sera of NC and $(GME)_p$ obtained by centrifugation from several samples were subjected to laser Raman spectrophotometer (Jasco, Model NR-1000) consisting of a photomultiplier unit (R-464) and an argon-ion laser at 488 nm, 80 mW having a scan speed of 5 $cm^{-1}\,s^{-1}$. An average of 4 scans was taken by the computer. Sensitivity was 5x kpps × 100 pA. Time constant was 0.5 s. Zero suppression was at 2.8. Interference filter was used. ν expansion was at 10 $cm^{-1}\,div^{-1}$.

The following characteristics have been observed in a typical spectrum (Figure 2) of NC serum: (a) strong Raman bands at 1534 cm^{-1} (ν_6) and 1160 cm^{-1} (ν_3) corresponding to C=C and C–C stretching vibrations respectively due to carotenoids and a moderately strong band at ~ 1010 cm^{-1} (ν_2) corresponding to $C-CH_3$

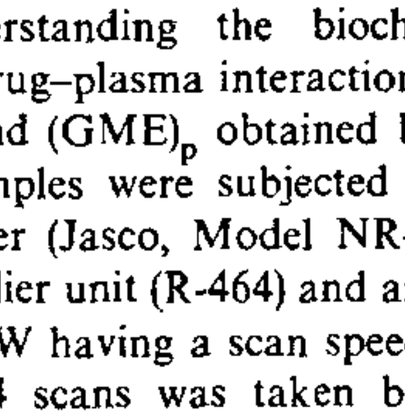

Figure 1. 5,5-diphenylhydantoin.