Proton Resonance Assignments with the Aid of Paramagnetic Relaxation Reagents

By G. Victor Fazakerley * and Graham E. Jackson, Department of Inorganic Chemistry, University of Cape Town, Rondebosch 7700, South Africa

In the presence of certain paramagnetic ions the transverse relaxation times (T_2), readily available from half-width measurements, provide a sensitive probe to molecular structure. Protons closest to the metal ion are preferentially broadened and this is used to assign resonances.

PARAMAGNETIC relaxation reagents have been in considerable use in biochemistry [1] for some time but their application to small organic molecules has been limited. A recent paper [2] has suggested that the use of organic-soluble reagents such as $Fe(acac)_3$ and the measurement of spin-lattice relaxation times (T_1) could complement lanthanide shift reagents as an aid to n.m.r. analysis. We report the use of some relaxation reagents in aqueous solution involving the measurement of transverse relaxation times (T_2).

Although the theory of line broadening has been known for many years the application of these relaxation reagents is not altogether straightforward. In principle the strong local fields produced by unpaired electrons can be coupled to a nucleus by simple dipole–dipole interactions or by a scalar or hyperfine coupling transmitted through chemical bonds. The Solomon [3] and Bloembergen [4] equations for the electronic contributions to the relaxation times are (1) and (2) where γ_I is the nuclear

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{g^2\gamma_I^2\beta^2S(S+1)}{r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right] + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left[\frac{\tau_e}{1+\omega_s^2\tau_e^2} + \tau_e\right] \quad (1)$$

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{g^2\gamma_I^2\beta^2S(S+1)}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right] + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left[\frac{\tau_e}{1+\omega_s^2\tau_e^2}\right] \quad (2)$$

magnetogyric ratio, β the Bohr magneton, ω_I and ω_s the nuclear and electronic Larmor frequencies, S the electronic spin quantum number, A the hyperfine coupling constant, τ_c and τ_e the dipolar and scalar correlation times, and r the metal ion–proton internuclear distance.

Accurate T_1 measurements are not readily available in many laboratories and we have considered what use can be made of T_2 measurements that are obtainable from line-width observations.

In order that we may make use of these equations it is necessary to simplify them which may be done if the following conditions are met. (i) That the second term (the scalar contribution) in equation (1) is negligible. (ii) That the condition of extreme narrowing holds ($\omega_I^2\tau_c^2 \ll 1$). (iii) That τ_c, which may be resolved into expression (3) is determined by the value of τ_r (τ_s and τ_r

$$\frac{1}{\tau_c} = \frac{1}{\tau_s} + \frac{1}{\tau_m} + \frac{1}{\tau_r} \quad (3)$$

are the electronic and rotational correlation times, τ_m the correlation time for chemical exchange).

The first condition has been generally found to be met if Cu^{II}, Mn^{II}, and Gd^{III} are used as broadening agents.[5,6] This can be checked if both T_1 and T_2 measurements can be made or if, as in this present work, T_2 measurements are made on a simple model system. The second condition is met if relatively small molecules are being studied. In this work τ_c *ca.* 10^{-11} s and $\omega_I^2\tau_c^2$ *ca.* 10^{-4}. The third condition is satisfied if τ_r is the shortest correlation time. For small molecule systems and these paramagnetic ions τ_m, $\tau_s > 10^{-9}$ s, while τ_r *ca.* 10^{-11} s.

A much more detailed analysis of the applicability of the Solomon and Bloembergen equations has been presented by Dwek.[1]

One further condition must be satisfied in order to be able to determine T_{2M} directly from the observed line-width. Swift and Connick [7] have related the observed line-width to T_{2M} and the rate of chemical exchange [equation (4) where T_{2obs} is the observed relaxation time,

$$\frac{1}{T_{2p}} = \frac{1}{T_{2obs}} - \frac{1}{T_{2(0)}} = \frac{f}{T_{2M} + \tau_m} \quad (4)$$

$T_{2(0)}$ is the relaxation time in the absence of paramagnetic ion, and f the ratio of paramagnetic ion concentration to ligand concentration].

To determine T_{2M} from T_{2obs}, T_{2M} must be large compared to τ_m. This is the condition of fast chemical exchange which may be readily tested for. The dependence of line-width upon the rate of chemical exchange is well known.[8] Thus if a plot of $(fT_{2p})^{-1}$ against reciprocal temperature shows a positive slope fast exchange conditions hold (Figure 2).

Equation (1) can now be simplified to (5) where K is a

$$\frac{1}{T_{2p}} = \frac{f}{T_{2M}} = K\frac{f}{r^6} \quad (5)$$

[1] R. A. Dwek, 'Nuclear Magnetic Resonance in Biochemistry,' Clarendon Press, Oxford, 1973.
[2] G. C. Levy and R. A. Komoroski, *J. Amer. Chem. Soc.*, 1974, **96**, 678.
[3] I. Solomon, *Phys. Rev.*, 1955, **99**, 559.
[4] N. Bloembergen, *J. Chem. Phys.*, 1957, **27**, 572.
[5] R. A. Dwek, O. W. Howarth, D. F. S. Natusch, and R. E. Richards, *Mol. Phys.*, 1967, **13**, 457.
[6] H. Sternlicht, R. G. Shulman, and E. W. Anderson, *J. Chem. Phys.*, 1965, **43**, 3133.
[7] T. J. Swift and R. E. Connick, *J. Chem. Phys.*, 1962, **37**, 307.
[8] T. A. Pople, W. G. Schneider, and H. J. Bernstein, 'High-resolution Nuclear Magnetic Resonance Spectroscopy,' McGraw-Hill, New York, 1959.

constant during the experiment. Qualitatively this means that the protons closest to the paramagnetic ion will be preferentially broadened and that this is highly sensitive because of the sixth power function. Quantitatively, distances can be compared by taking the slopes for two protons from plots of $(T_{2p})^{-1}$ against f [equation (6)]. In organic solvents there is an added

$$\frac{\text{slope}_1}{\text{slope}_2} = \left(\frac{r_2}{r_1}\right)^6 \qquad (6)$$

complication of inner and outer sphere co-ordination which makes distance evaluation less reliable. In aqueous solution where a good binding site is available for the metal ion, outer sphere co-ordination is negligible and quantitative results can be obtained. If the site of co-ordination is known, n.m.r. assignments can be made from observed T_2 values.

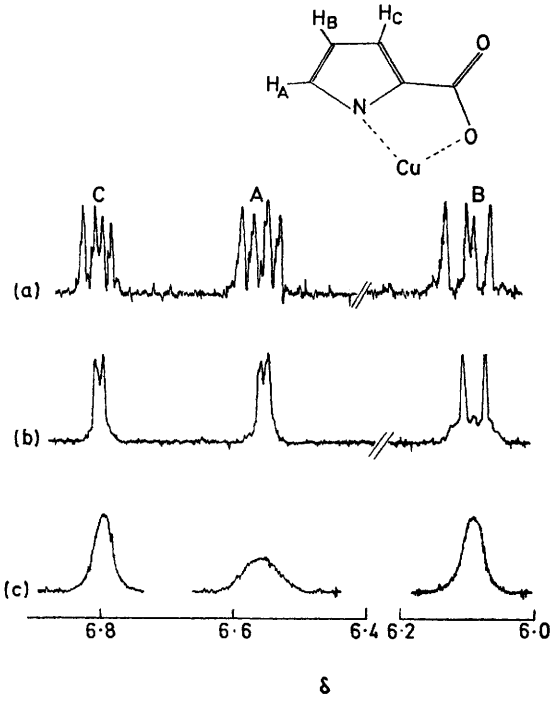

FIGURE 1 (a) ^{1}H N.m.r. spectrum of 0·1M-pyrrole-2-carboxylic acid, pH 5·5, 25°; (b) partially spin-decoupled spectrum, A and C from B, B from C; (c) (b) + 1·6 × 10^{-5}M-Mn^{2+}

RESULTS AND DISCUSSION

To demonstrate the validity of this technique we have studied three systems of increasing complexity. The first is pyrrole-2-carboxylic acid which has only one site and one possible conformation. The second is thiaproline which has only one site but two possible conformations. The third is benzylpenicillin which has several sites and several possible conformations.

Pyrrole-2-carboxylic acid–Mn^{2+}.—The spectrum of pyrrole-2-carboxylic acid is shown in Figure 1(a) with the assignment based on coupling constant data.[9] The partially decoupled spectrum (A and C from B, B from C) [Figure 1(b)] was then broadened by the addition of Mn^{2+} [Figure 1(c)]. A variable temperature study on the partially broadened spectrum is shown in Figure 2. This confirms that we are working with fast chemical exchange. Figure 3 shows the broadening observed for the three protons during the titration. Table 1 gives the ratio of the experimentally observed internuclear distances compared to those measured from a Dreiding

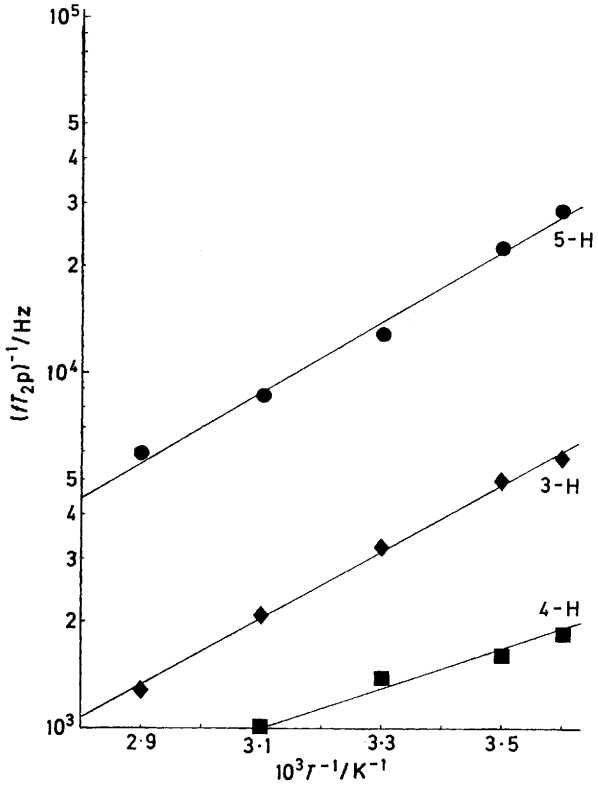

FIGURE 2 Temperature dependence of fT_{2p} for pyrrole-2-carboxylic acid–Mn^{2+}, pH 5·5

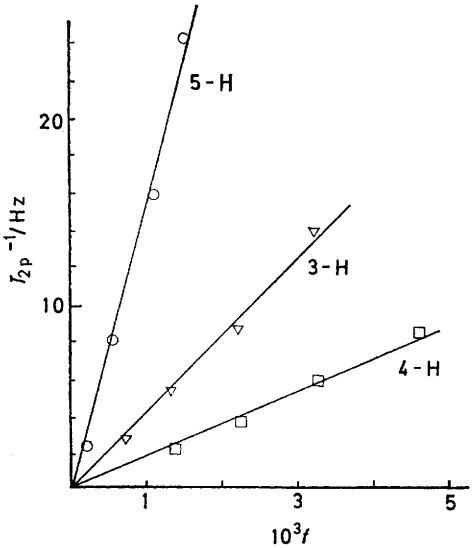

FIGURE 3 Measured values of T_{2p} for pyrrole-2-carboxylic acid–Mn^{2+} as a function of f, pH 5·5, 25°

[9] H. Erlenmeyer, R. Griesser, B. Prijis, and H. Sigel, *Helv. Chim. Acta*, 1968, **51**, 338.

model of the complex. The Mn^{2+} ion is assumed to be in the same plane as the pyrrole ring. The agreement

TABLE 1

Comparison of measured and experimentally determined proton–metal distances for pyrrole-2-carboxylic acid–Mn^{2+}

Proton	Measured (Å)	Experimental (Å) [a]
3-H	4·7	4·6
4-H	5·2	5·3
5-H	3·6	3·6

[a] Relative to 3-H.

between measured and experimentally observed distances is within 6% a result of the sixth power of r in equation (4). A 60% error in the relative slopes produces a 10% error in the relative distances.

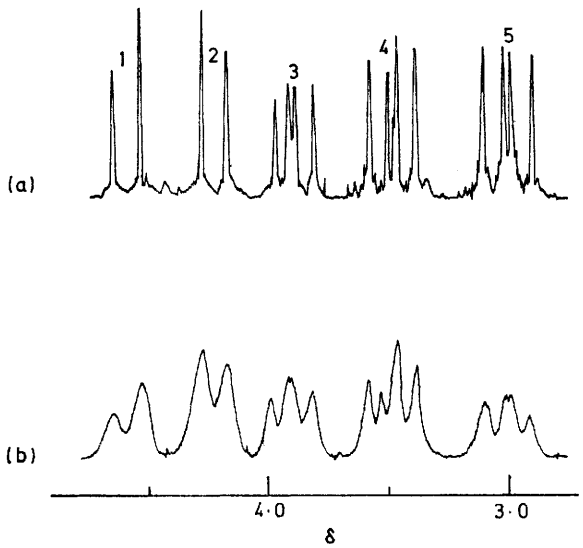

FIGURE 4 ^1H N.m.r. spectrum of thiaproline in the presence and absence of Mn^{2+}: (a) 0·1M-thiaproline, pH 8·0, 25 °C; (b) as (a) + 4 × 10^{-5}M-Mn^{2+} J_{12} 9·5, J_{43} 8·2, J_{45} 10·5, J_{53} 7·2 Hz

Qualitatively it can be seen from the model that the r values are in the order A < C < B. Examination of the single partially broadened spectrum in Figure 1 (c) allows assignment of the resonances.

Thioproline–Cu^{2+} or –Mn^{2+}.—The spectrum of thiaproline itself and the partially broadened spectrum as shown in Figure 4. 1- and 2-H give rise to an AB pattern while 3—5-H give an ABX pattern. The analysis of the spectrum was according to the method of Diehl [10] and is as indicated.

The temperature dependence of T_{2p} showed that we were in the region of fast chemical exchange, and from a plot of $1/fT_{2p}$ against $1/T$ the activation energy for rotation can be calculated (Table 2). This is found to be similar to that for $Mn(H_2O)_6^{2+}$.[11]

Thus we may now interpret the metal ion titration plot (Figure 5) to give relative internuclear distances in the complex. Although there is only one possible binding site the thiaproline ring may assume two conformations (Figure 6). Although the five-membered ring involving the metal ion will presumably flip it need not be considered here as the position of the metal relative to

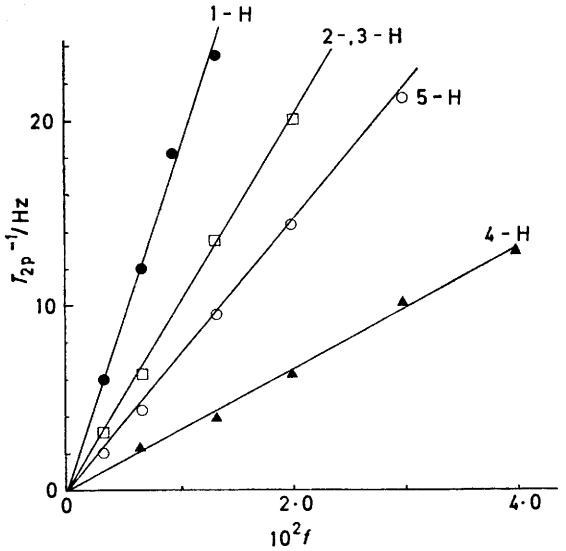

FIGURE 5 Measured values of T_{2p} for thiaproline–Mn^{2+} as a function of f, pH 8·0, 25°

the thiaproline protons is unchanged. The measured internuclear distances for the two conformations together with the experimentally observed values are given in Table 3. We cannot distinguish a preference for one of the two conformations.

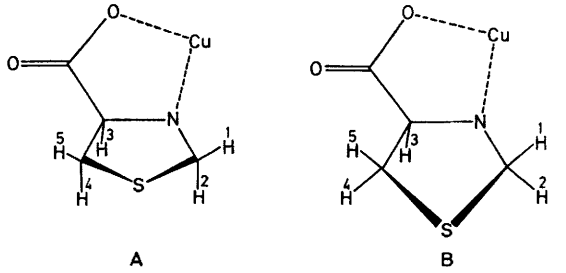

FIGURE 6 Schematic drawing of the Mn^{2+}–thiaproline complex showing the two possible conformations

TABLE 2

Parameters obtained from analysis of relaxation rates in the Mn^{II}–thiaproline system

a (effective radius) (Å)	4·0
E_A/kcal mol^{-1}	4·6
τ_r (298 K) (s)	6·5 × 10^{-11}
τ_r (298 K) (s) $Mn(H_2O)_6^{2+}$ [11]	3 × 10^{-11}
E_A/kcal mol^{-1} $Mn(H_2O)_6^{2+}$ [11]	4·5

Qualitatively it is easy to assign the n.m.r. spectrum. From the model it is clear that at all times 1-H is closer to the metal than 2-H. Thus 1-H will broaden faster than 2-H (see Figure 4). 1-H May be assigned to the low-field resonance. Similarly 4- and 5-H may be assigned to the low- and high-field resonances respectively.

[10] P. Diehl, *Helv. Chim. Acta*, 1945, **48**, 567.
[11] N. Bloembergen and L. O. Morgan, *J. Chem. Phys.*, 1961, **34**, 842.

TABLE 3

Comparison of measured and experimentally determined proton–metal distances for thiaproline–Mn^{2+} and Cu^{2+} systems

Proton	Measured distances Å for two conformations A	B	Experimental [a] distance Mn^{2+}/Cu^{2+} (Å)
H_1	3·0	2·7	3·1
H_2	3·3	3·8	3·6
H_3	3·6	3·5	3·5
H_4	4·8	4·7	4·3
H_5	3·9	4·7	3·7

[a] Relative to 3-H.

These are in agreement with our own assignment from coupling constant data.

Benzylpenicillin–Cu^{2+} *or* –Mn^{2+}. The spectrum of benzylpenicillin is shown in Figure 7. Cooper [12] has

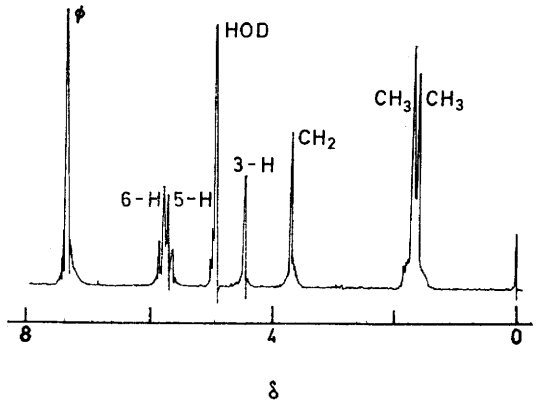

FIGURE 7 ^1H N.m.r. spectrum of benzylpenicillin, 0·1M, pH 5·5, 25°

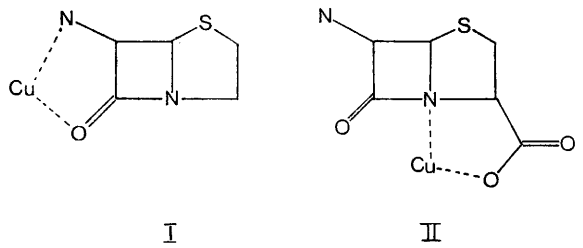

FIGURE 8 Schematic drawing of the two most likely structures of the Cu^{2+}–benzylpenicillin complex

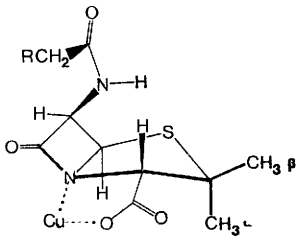

FIGURE 9 Schematic drawing of the Cu^{2+}–benzylpenicillin complex in solution, showing the B conformation of the thiazolidine ring and the orientation of the side-chain

assigned the spectrum in deuteriochloroform solution. The two methyl groups were assigned from NOE experiments and 5- and 6-H from coupling with the amine proton. Such an assignment is not possible in aqueous solution. However our experiments show that the assignment is the same in aqueous solution.

The variable temperature experiments show that we are in the region of fast exchange. With Cu^{2+} but not with Mn^{2+} rapid hydrolysis occurs at about 340 K consistent with the observations of Cressman [13] that Cu^{2+} specifically promotes hydrolysis of penicillins. We expect the rotational correlation time (*ca.* $6 \cdot 10^{-10}$ s) to modulate relaxation.

There are several possible sites of co-ordination for the metal ion on penicillin and the two perhaps most likely are shown in Figure 8. From kinetic evidence Cressman [13] proposed structure (I) although this does not preclude the possibility of there being more than one structure in solution with (II) as an alternative perhaps even predominating.

No appreciable broadening occurred on the side-chain methylene whereas 3-H broadened rapidly. Co-ordination involving the side-chain was rejected. It may occur but to a very small degree. The alternative of co-ordination through the ring nitrogen and the carboxy-group fits the experimental data if we assign 5- and 6-H to the high- and low-field resonances respectively. Using the reverse assignment we are unable to find any meaningful site of co-ordination.

Considering the two methyl groups on the thiazolidine ring, whichever conformation the ring has α-CH_3 will always be closer to the metal than β-CH_3. Thus the high-field signal can be assigned to α-CH_3.

Table 4 gives the calculated and observed internuclear

TABLE 4

Comparison of measured and experimentally determined proton–metal distances for benzylpenicillin–Mn^{2+} and Cu^{2+} systems

Proton	Measured distances (Å) for two conformations [a] A	B	Experimental [b] distance Mn^{2+}/Cu^{2+} (Å)
3-H	3·2	3·6	3·4
5-H	2·8	2·8	3·0
6-H	4·1	4·1	4·1
α-CH_3	4·5	2·7	3·5
β-CH_3	5·0	4·5	4·3
CH_2			5·8

[a] Relative to 3-H. [b] Conformation A has α-CH_3 equatorial.

distances for the two conformations of the thiazolidine ring. It is possible that conformation B is slightly favoured over A. From the broadening of the methylene we position the side-chain as in Figure 9.

Added confidence in the assignments comes from the use of two probes, Mn^{2+} and Cu^{2+}, which give the same results. We therefore propose that the hydrolysis of penicillins by Cu^{2+} occurs through some other structure too minor to be detected by this technique.

General Conclusions.—We have demonstrated the power of paramagnetic broadening agents in the assignments of proton resonances, in the cases of pyrrole-2-

[12] R. D. Cooper, P. V. Demarco, J. C. Cheng, and N. D. Jones, *J. Amer. Chem. Soc.*, 1969, **91**, 1408.

[13] W. A. Cressman, E. T. Sugita, J. T. Doluisio, and P. J. Niebergal, *J. Pharm. Sci.*, 1969, **58**, 1471.

carboxylic acid, thiaproline, and benzylpenicillin. This is particularly easy where a single chelate structure can be proposed for the complex. It is particularly suitable for small molecule systems. For small molecules τ_r is 10^{-10}—10^{-11} s, whereas for macromolecules τ_r is 10^{-7} s. For Mn^{2+} τ_s is 10^{-8} s and in macromolecule system relaxation is often dominated by τ_m. However considerable care has to be taken to ensure that the various conditions, particularly that of fast chemical exchange, are satisfied.

EXPERIMENTAL

Pyrrole-2-carboxylic acid, thiaproline, and sodium benzylpenicillin were all commercial products. AnalaR $Mn(ClO_4)_2$, $6H_2O$ ($8 \cdot 10^{-3}$M), and $Cu(ClO_4)_2, 6H_2O$ ($4 \cdot 10^{-2}$M) solutions were prepared in D_2O and adjusted to pH 5·5 with NaOD or $DClO_4$. These solutions were used as the metal ion source.

0·1M-Solutions of the ligands in D_2O were used and small volumes of the metal ion solutions added by a microsyringe. In the benzylpenicillin–Cu^{2+} experiments the solutions were freshly prepared to minimise hydrolysis.

Spectra were recorded on a Varian XL-100 spectrometer. Care was taken to avoid saturation effects and samples were kept for 10 min to attain thermal equilibrium before spectra were recorded. The transverse relaxation times, T_2, were estimated from the line-widths ($\Delta\gamma$) at half peak height ($T_2^{-1} = \pi\Delta\gamma$). The analysis of the AB quartet of 5- and 6-H of benzylpenicillin was carried out using a computer simulated least squares fit for the observed curves. For methyl groups the calculated internuclear distance is given by equation (7).

$$\frac{1}{r_{av}^6} = \frac{1}{3}\left(\frac{1}{r_1^6} + \frac{1}{r_2^6} + \frac{1}{r_3^6}\right) \quad (7)$$

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