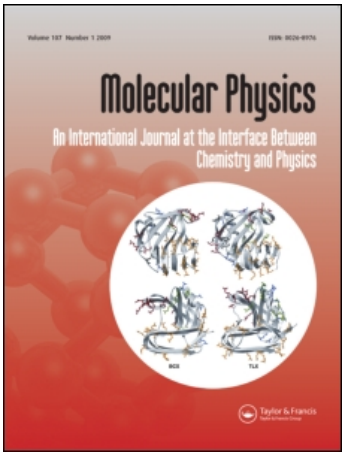

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Proton magnetic relaxation and molecular motion in polycrystalline amino acids

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Proton magnetic relaxation and molecular motion in polycrystalline amino acids
II. Alanine, isoleucine, leucine, methionine, norleucine, threonine and valine

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A proton magnetic relaxation study has been carried out on a further seven polycrystalline amino acids from 130 to 500 K at 60·2 MHz, supplemented by measurements of the spectrum down to 4 K on five of them. These amino acids all have one or two methyl groups in their side chain, and exhibit two relaxation minima. Clear evidence is given that the relaxation minima at lower temperatures are attributable to reorientation of the methyl groups, while those at higher temperatures are attributable to reorientation of the $-NH_3$ groups in the zwitterion configuration of the molecules. Values of the relaxation constants, activation energies and time factors which best characterize the kinetics of both motions are tabulated. Effects of methyl group tunnelling are found for methionine and valine.

1. INTRODUCTION

In a preceding paper [1] (to be referred to as I) an account was given of the proton spin–lattice relaxation and molecular motion in the solid state for seven of the amino acids commonly encountered in proteins. In all seven the side group R in the molecule $^{+}H_3N–CHR–COO^{-}$ contained no molecular rotor, and the mechanism of relaxation was ascribed to reorientation of the $-NH_3$ group in the zwitterion structure. In this paper we consider a second group of seven amino acids, all of which include one or more methyl groups in the side chain R (see table 1), and all show evidence of independent relaxation through the $-NH_3$ and $-CH_3$ groups, which are separately identified.

2. EXPERIMENTAL DETAILS

Four of the compounds, namely, alanine, isoleucine, threonine and valine, were in the L form, and the other three, leucine, methionine and norleucine, were in the DL form. The preparation of specimens and the details of the nuclear magnetic resonance measurements were described in I. Work was also

Amino acid	Side chain	Number of protons n	R.M.S. (obs–calc) per cent	Reorienting group	T_1 min at 60 MHz ms	Temp. at T_1 min K	Relaxation constant C 10^8 s^{-2}	Activation energy E kJ/mole	Time factor τ_0 10^{-14} s
L-alanine	$-CH_3$	7	5·6	$-NH_3$	61	402	43·5	38·6	1·6
				$-CH_3$	76	289	35·0	22·4	15
L-alanine d_3	$-CH_3$	4	2·9	$-CH_3$	72	277	37·0	22·5	9
L-isoleucine	$-CH(CH_3)CH_2CH_3$	13	6·7	$-NH_3$	116	432	22·8	44·8	0·6
				$-CH_3$				13·0	
DL-leucine	$-CH_2CH(CH_3)_2$	13	4·2	$-NH_3$	120	454	22·2	51·7	0·2
				$-CH_3$	97	200	27·4	13·2	59
DL-methionine	$-CH_2CH_2SCH_3$	11	5·6	$-NH_3$	105	376	25·2	39·4	0·5
				$-CH_3$				6·7	
DL-norleucine	$-(CH_2)_3CH_3$	13	2·4	$-NH_3$	114	375	23·2	41·7	0·25
				$-CH_3$	149	164	17·8	12·6	16
L-threonine	$-CH(CH_3)OH$	9	4·1	$-NH_3$	78	362	34·2	32·5	3·3
				$-CH_3$	109	179	24·4	12·0	50
L-valine	$-CH(CH_3)_2$	11	10·2	$-NJ_3$	108	422	24·5	37·4	3·8
				$-CH_3$	111	201	24·0	11·3	189

Table 1. Relaxation parameters for the molecular motion of the NH_3 and CH_3 groups.

done on the partially deuterated species of L-alanine, $^{+}D_3NCH(CH_3)COO^{-}$. This species was prepared by repeated crystallization of alanine from D_2O. The progress of deuteration was monitored by high resolution proton N.M.R.

3. Results and analysis

The measured values of proton spin–lattice relaxation time T_1 are shown as a function of temperature in figures 1 to 4. All the measurements were made at 60·2 MHz except for L-valine, for which measurements were also made at 38·8 MHz. In each of the figures the full line is a calculated theoretical line. Five of the amino acids exhibit two clearly resolved minima, while in the remaining two there is clear evidence of a second relaxation process effective at lower temperatures, although the second minimum was not reached.

The existence of two minima in the variation of T_1 with temperature suggests that the nuclear relaxation is generated by the random reorientation of two distinct molecular rotors within the solid. The results have therefore been analysed using the following relaxation expression :

$$T_1^{-1} = \sum_i C_i[\tau_i(1+\omega^2\tau_i^2) + 4\tau_i(1+4\omega^2\tau_i^2)^{-1}]. \quad (1)$$

This is an extension of the well-known relaxation expression of Kubo and Tomita [2] to several independent relaxation processes, assuming no correlation or interference between them. In equation (1) $\omega/2\pi$ is the N.M.R. frequency of measurement, and C_i and τ_i are the relaxation constants and correlation times of the random molecular motions responsible for spin–lattice relaxation. The

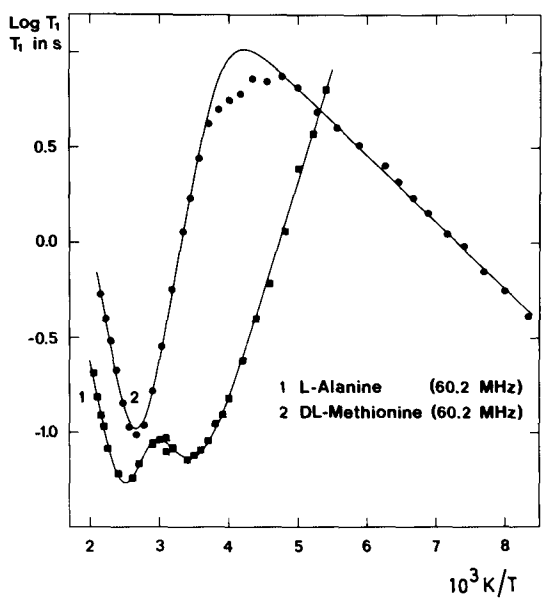

Figure 1. The variation of proton spin–lattice relaxation time T_1 with inverse temperature T^{-1} for polycrystalline amino acids at 60·2 MHz. Curve 1 : L-alanine, curve 2 : DL-methionine. The full lines are theoretical curves calculated in the manner described in the text.

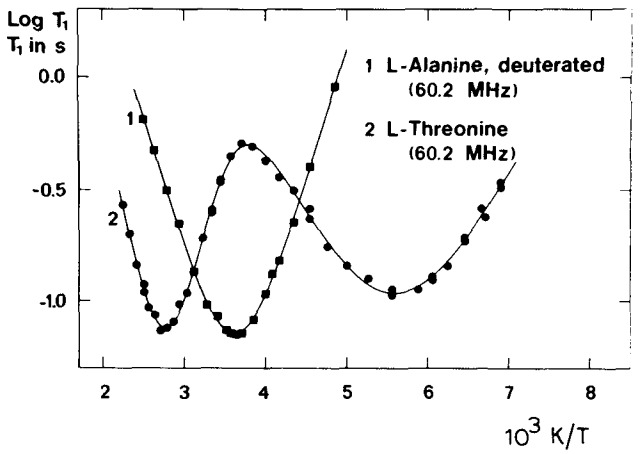

Figure 2. The variation of proton spin–lattice relaxation time T_1 with inverse temperature T^{-1} for polycrystalline amino acids at 60·2 MHz. Curve 1 : L-alanine-d_3, curve 2 : L-threonine. The full lines are theoretical curves calculated in the manner described in the text. Note that deuteration of the NH_3 group in L-alanine has removed the high-temperature minimum (figure 1).

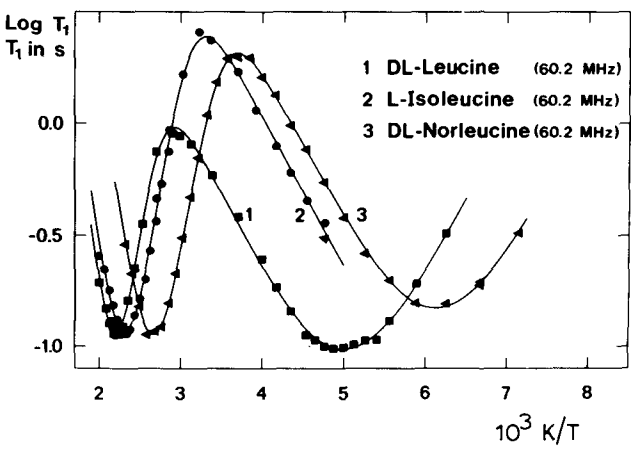

Figure 3. The variation of proton spin–lattice relaxation time with inverse temperature T^{-1} for polycrystalline amino acids at 60·2 MHz. Curve 1 : DL-leucine, curve 2 : L-isoleucine, curve3 : DL-norcleucine. The full lines are theoretical curves calculated in the manner described in the text.

analysis further assumed that the correlation time of each motion followed a simple activation law

$$\tau_i = \tau_{0i} \exp E_i/kT. \tag{2}$$

A computer programme minimized the r.m.s. percentage difference between observed and calculated values of T_1 refining simultaneously the parameters characterizing each of the two relaxation processes. The best values of C, τ_0 and E for each process are listed in table 1 and the full lines in figures 1 and 4 were calculated from equations (1) and (2) using these values. In the case of

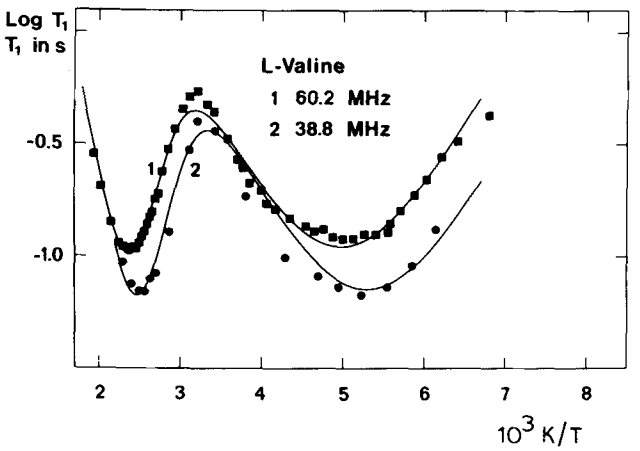

Figure 4. The variation of proton spin–lattice relaxation time T_1 with inverse temperature T^{-1} for polycrystalline L-valine. Curve 1 : 60·2 MHz, curve 2 : 38·8 MHz. The full lines are theoretical curves calculated in the manner described in the text.

L-valine the data at 60·2 and 38·8 MHz were refined together and the two calculated theoretical curves are based on the same values of C, τ_0 and E.

For alanine, isoleucine, leucine, norleucine and threonine the agreement between the measurements and the theoretical curves calculated on the assumption of two relaxation processes is excellent over the whole range. For methionine the agreement is very good over most of the range except in the vicinity of the maximum of T_1 at 240 K, where the experimental points fall below the calculated curve. This may suggest a third, weaker, relaxation mechanism effective here, the possibility of which is discussed later. The points around the maximum were therefore not included in the final computer fitting. For valine there is good general agreement over the whole range for both measuring frequencies, but the detailed agreement is not quite as good as in the other cases, particularly below 350 K, the r.m.s. deviation (table 1) being about twice that of other compounds. Here too the simple assumption of just two independent relaxation processes requires examination. We note from equation (1) that at higher temperatures, where $\omega^2\tau_i^2 \ll 1$, the relaxation time is proportional to τ_i^{-1} and is independent of frequency, and we see for valine (figure 4) that on the high temperature side of each minimum the curves and sets of measurements for the two frequencies come together. On the other hand at lower temperatures, where $\omega^2\tau_i^2 \gg 1$, T_1 is proportional to $\omega^2{}_{2i}$, also in agreement with experiment ; furthermore, the value of the relaxation time at each minimum, $T_{1\,\mathrm{min}}$, is proportional to frequency, in agreement with (1).

The two motional processes responsible for relaxation also manifest themselves in the narrowing of the N.M.R. spectra with increasing temperature. The second moment of the proton magnetic resonance spectra is shown as a function of temperature for two of this group of amino acids in figures 5 and 6, and is seen to fall in two successive steps as the frequency af the motion of each process becomes comparable with the spectral width. Plateau values of the second moment, accuracy about 5 per cent, are listed in table 2.

Also shown in table 2 are computed theoretical values of second moment of the spectrum calculated using the well-known expression of Van Vleck [3]

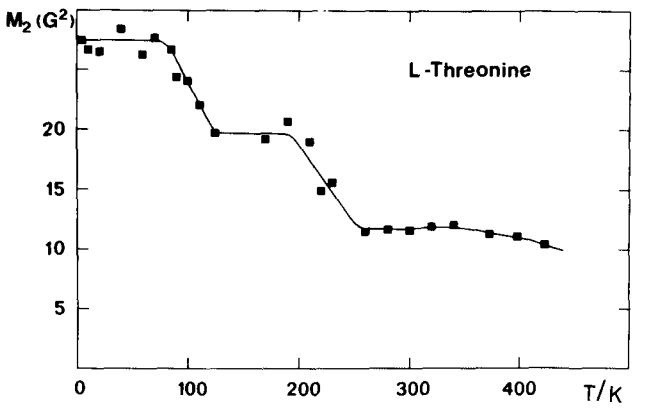

Figure 5. The variation of second moment M_2 of the proton magnetic resonance spectrum with temperature T for polycrystalline L-threonine.

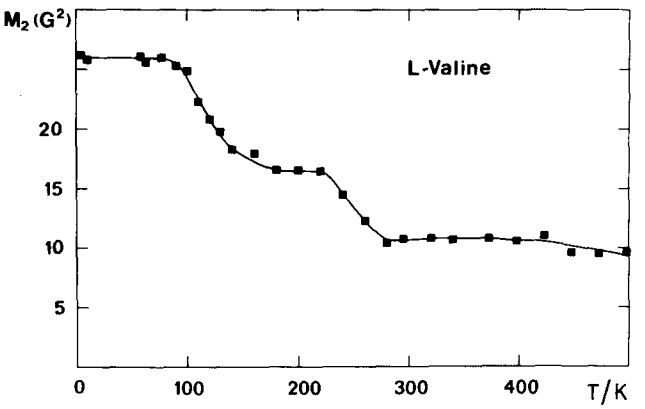

Figure 6. The variation of second moment M_2 of the proton magnetic resonance spectrum with temperature T for polycrystalline L-valine.

Amino acid	Measured plateau values/G^2			Calculated M_2/G^2 Rigid	CH_3 Rot	CH_3 and NH_3 Rot	T_n CH_3	NH_3
Isoleucine	30	20	11	32·1	20·8	13·8		265
Leucine	26	18·5	8	31·2	20·7	13·7	100	290
Methionine	21			27·4	21·9	13·8		230
Threonine	27·5	20	11·5	28·3	21·2	11·0	90	210
Valine	26	17	11	33·0	20·0 (all) 26·5 (A only) 26·2 (B only)	11·7	100	240

Table 2. Plateau values of second moment M_2.

	References
L-alanine	[13, 16]
L-isoleucine	[17]
DL-leucine	[18]
DL- and L-methionine	[19, 20]
L-norleucine	[20]
L-threonine	[21]
L-valine	[15]

Table 3. X-ray and neutron-diffraction references.

and the procedure of Sjöblom [4]. For this purpose the proton coordinates in the crystal lattice were projected from the X-ray diffraction data (table 3) assuming tetrahedral angles and C–H and N–H bond lengths of 1·10 Å and 1·04 Å respectively [5, 6]. We expect the dipolar averaging due to molecular reorientation to narrow the spectrum when τ_i is the same order as $(2\pi\delta\nu)^{-1}$, where $\delta\nu$ is the linewidth [7]. The spectra are of the order 10 G wide, and using equation (2) for τ_i together with the values of E and τ_0 from table 1 the temperatures T_n at which the spectra may be expected to narrow have been calculated and are given in table 2.

When comparing the values of activation energy E in table 1, where it is possible, with those of other workers, it has to be borne in mind that our data have been fitted over the whole range of temperature. Other workers have generally based their values on the limiting slope of the graph which plots ln T_1 against T^{-1}; as equations (1) and (2) show, this gives E from a linear plot provided $\omega^2\tau^2 \ll 1$ or $\gg 1$. This method tends to give lower values of E, since the data may not be sufficiently far from the minimum to meet the requirements of the inequalities strongly enough, and since long values of T_1 are stressed in the evaluation and these are more affected by secondary relaxation processes. Thus for the high temperature process our values of E from table 1 for alanine is 39 kJ/mole compared with 35 kJ/mole from Zaripov [8]. For DL-norleucine the respective values are 42 and 38 kJ/mole. Goren and Knispel [9] obtained the value 44 kJ/mole for L-isoleucine from the slope on the low temperature side of the high temperature minimum, in good agreement with our value of 45 kJ/mole, but for L-isoleucine their value of 29 kJ/mole is significantly less than our value of 45 kJ/mole based on the whole curve.

For L-alanine comparison may be made between our results on the normal compound and on the deuterated version $^+D_3NCH(CH_3)COO^-$ for which the high temperature proton relaxation process is effectively suppressed (figures 1 and 2), isolating the low temperature process for more direct evaluation. As table 1 shows, the values of E for the normal and deuterated species were 22·4 and 22·5 kJ/mole, agreeing very well within the estimated uncertainty of ± 1 kJ/mole, and implying that deuteration, as expected, had little affected the constraints imposed on the methyl groups.

Some evidence of non-exponential recovery of magnetization was noted in the case of L-alanine, dependent on the particular pulse sequence used. Such non-exponential behaviour has been noticed by other workers for triangular

rotor systems and has been explained in terms of the correlated motion of the three-spin system and the coupling between the nuclear magnetization and the rotational polarization [10, 11].

4. Discussion

X-ray and neutron-diffraction investigations, listed in table 3, give strong evidence that these seven amino acids are in the zwitterion form in the crystalline state. In the first group of seven amino acids discussed in I it was concluded that reorientation of the $-NH_3$ group provided the main relaxation mechanism, and we may therefore expect that this motion will provide one important relaxation mechanism for this second group of seven also. This second group also all exhibit a second relaxation mechanism. Since this group of amino acids all include one or more methyl groups in the side chain (table 1), whereas those in I did not, we may expect that reorientation of the $-CH_3$ groups provides the second relaxation mechanism. We next have to decide which molecular rotor is responsible for the low temperature relaxation process and which is responsible for that at high temperatures.

Since the $-NH_3$ groups participate in hydrogen bonds which have an important role in the structure and cohesion of the crystal, whereas the $-CH_3$ groups do not, we may expect the methyl groups to be more free to rotate and to be responsible for the lower temperature relaxation processes. This is reinforced by the observation for alanine and threonine, which both have one $-NH_3$ group and one $-CH_3$ group that the value of $T_{1\,\mathrm{min}}$ for the lower temperature minimum is significantly higher than that for the higher temperature minimum. An explanation of this observation is to be found in the fact that the C–H bond length is some 5 per cent longer than the N–H bond length, and its dipolar interaction is correspondingly weaker. Moreover, in I it was found for $-NH_3$ reorientation that C^{-1}, where C is the relaxation constant in equation (1), is proportional to n, the number of protons in the amino acid molecule, with nC close to $30 \times 10^9\ \mathrm{s}^{-2}$. For all the seven amino acids in this paper the values of the product nC for the higher temperature relaxation process (table 1) fall in the range 27 to $31 \times 10^9\ \mathrm{s}^{-2}$. For the lower temperature relaxation process the values of the product nC fall outside this range. Thus for alanine and threonine, which have one methyl group, the nC values for the low temperature process are 24 and $22 \times 10^9\ \mathrm{s}^{-2}$ respectively. Finally the most cogent evidence of all is from the comparison of the spin–lattice relaxation in L-alanine and in L-alanine-d_3 ($^+D_3NCH(CH_3)COO^-$), figures 1 and 2; deuteration of the NH_3 group has removed the high temperature minimum.

Having identified the higher temperature relaxation mechanism with the $-NH_3$ group reorientation and the lower temperature mechanism with methyl group reorientation we now turn to a more detailed examination of each amino acid in turn, starting with *L-alanine*. The ratio of the relaxation constants C for the $-NH_3$ and $-CH_3$ groups is $1{\cdot}24 \pm 0{\cdot}05$ (table 1). If we neglect the smaller contributions to relaxation arising from modulation of the weaker dipolar interactions between protons in one rotor and remoter protons external to the rotor, we may ascribe the ratio 1·24 to the stronger dipolar interactions between the protons in the NH_3 group compared with those in the CH_3 group. For three protons at the vertices of an equilateral triangle of side b, the values of C for

magnetic dipolar relaxation is given by [12]

$$C = \frac{9\gamma^4\hbar^2}{20b^6}. \tag{3}$$

The neutron-diffraction investigation of L-alanine by Lehmann *et al.* [13] shows that the protons in the CH_3 group are equilateral within experimental error with a mean separation of $1{\cdot}755 \pm 0{\cdot}005$ Å. For the NH_3 group the proton separations differ slightly on account of hydrogen bonding, but the differences are less than 1 per cent, the mean separation being $1{\cdot}686 \pm 0{\cdot}007$ Å. The ratio of relaxation constants expected from equation (3) is therefore $1{\cdot}27 \pm 0{\cdot}03$, in satisfactory agreement with the observed ratio of $1{\cdot}24 \pm 0{\cdot}05$, and further supports the correct identification of the two relaxation processes.

Threonine, like alanine, has one $-NH_3$ group and one $-CH_3$ group, and the ratio of relaxation constants (table 1) in this case is $1{\cdot}40 \pm 0{\cdot}05$. Making the same assumptions as for alanine, use of equation (3) gives a ratio of effective mean inter-proton distances of 1·057 in the two groups. Comparison with directly measured distances must await a neutron-diffraction investigation. However, taken with a C–H bond length of 1·10 Å the ratio leads to a mean N–H bond length of 1·04 Å, close to the mean value found in other amino acids [6]. The three observed plateau values of second moment (table 2) are in very satisfactory agreement with the calculated values for a rigid molecular array, for rapid CH_3 reorientation, and for rapid reorientation of both $-CH_3$ and $-NH_3$ groups, respectively. The temperatures T_n at which the spectrum is expected to be narrowed by these two motional processes (table 2) are also in excellent agreement with observation (figure 5).

Norleucine also has a single methyl group in its side chain R. The ratio of relaxation constants (table 1) for the two groups is $1{\cdot}30 \pm 0{\cdot}05$ (cf. 1·24 for alanine and 1·40 for threonine), and corresponds to a ratio of effective mean inter-proton distances of 1·045 in the two groups.

The only other amino acid in this group with a single methyl in the side chain is *methionine*, where it is connected to the rest of the molecule by a sulphur atom. The distinctive feature of the relaxation behaviour for this solid is the unusually low value 6·7 kJ/mole of E for the low temperature relaxation process (table 1), reflecting the larger distances between the methyl group and its neighbours. As a consequence we were not able to reach the low temperature minimum (figure 1) with the cryostat available for relaxation measurements, but from the parameters which best fit the relaxation data a minimum is predicted at about 88 K. For a hindered rotor in its lowest torsional oscillator state in a three-fold sinusoidal potential well, the tunnelling rate exceeds 10^5 Hz when the height of the barrier is less than 12 kJ/mole [14]. Though the potential barrier best describing the constraints under which the methyl group moves in solid methionine may not be precisely sinusoidal, the activation energy E is sufficiently low that we must expect that the proton N.M.R. spectrum will be narrowed by methyl group tunnelling even at the lowest temperatures. The low temperature plateau value of second moment (table 2) is in fact much lower than the calculated rigid-lattice value, but is in good agreement with that calculated for fast methyl group reorientation. The spectrum narrows further around the temperature T_n predicted for the effect of $-NH_3$ group reorientation (table 2). After falling to 14 G^2, the value predicted for combined fast $-CH_3$

and $-NH_3$ group reorientation, the experimental second moment continues to decrease, though more slowly, reaching 5 G^2 at 480 K. The additional form of motion responsible for this further narrowing may also be responsible for the deviations of T_1, in the vicinity of the maximum at 240 K, from the best calculated curve based on equation (1) with just two relaxation processes (figure 1). Possible candidates are reorientational motion of the side group R or of the whole molecule.

We now turn to the two isomers of norleucine, namely, leucine and isoleucine, both of which have branched side chains R containing two methyl groups. We note from table 1 that while the activation energies for the $-NH_3$ group motion for the three isomers are significantly different, the values for the methyl group motion are the same within experimental error.

Since the relaxation data for *leucine* (figure 3) are well accounted for by just two relaxation processes, it seems that the correlation times associated with reorientation of the two methyl groups in the molecule are closely the same and that their environments impose closely similar constraints. Since the ratios of relaxation constants for $-NH_3$ and $-CH_3$ groups for alanine, threonine and norleucine, all of which have just one methyl group, are 1·24, 1·40, 1·30 respectively, one might expect that for leucine with two similar methyl groups this ratio would be halved to the order of $0{\cdot}66 \pm 0{\cdot}04$. In fact the observed ratio (table 1) is 0·81, and the two methyl groups are evidently somewhat less than twice as effective in relaxation in leucine compared with the single methyl group in the other three amino acids.

The theoretical rigid-lattice second moment for leucine is significantly higher than the highest values recorded at temperatures down to 4 K (table 2). Perhaps a clue to these two small discrepancies is to be found by noting that the activation energy for methyl group reorientation in leucine is 13·2 kJ/mole (table 1). For a three-fold sinusoidal barrier whose height is 13·2 kJ/mole above the lowest torsional state, the tunnelling frequency in that state is 10 kHz [14]. The width of the proton spectrum, defined as the interval between derivative turning points, is 35 kHz below 100 K. Thus the tunnelling frequency, though less than the linewidth, is nevertheless comparable with it, and tunnelling effects may be sufficient to account for the small difference between the theoretical and observed second moments, and for the small reduction in relaxation efficiency. The predicted values of T_n are in satisfactory agreement with observation. Above 290 K the observed second moment falls to 10 G^2 at 340 K and then slowly to 6·5 G^2 at 500 K ; the ' plateau ' value of 8 G^2 in table 2 represents an average over this range. Additional motion, perhaps of the side chain, is evidently contributing in this range.

The low temperature relaxation minimum of *isoleucine* was not completed. Extrapolation, using plausible values of the relaxation parameters, suggests a minimum in the vicinity of 160 K. The three calculated values of second moment are in tolerable agreement with the three observed plateau values (table 2), though all about 2 G^2 higher. More reliable values of the theoretical second moment must await a neutron-diffraction determination of hydrogen positions in the crystal. The predicted line-narrowing temperature T_n agrees well with observation.

Finally we turn to *valine* which has some interesting features. The observed second moment below 80 K, based on good spectra, has the constant value

26 ± 1 G^2 (figure 4), much less than the calculated value for a rigid array, 33·0 G^2 (table 2), and yet much more than the calculated value for all methyl groups rapidly reorienting, 20·0 G^2. The crystal structure [15] shows an asymmetric unit with two crystallographically independent molecules with different molecular conformations. We have therefore also calculated the second moment on the assumption that only the methyl groups in one molecule are reorienting, obtaining the values 26·5 G^2 (methyls in molecule A rotating) and 26·2 G^2 (methyls in molecule B rotating). These two values are very close to the measured value, and we draw the provisional conclusion that the low temperature second moment is less than the rigid-lattice value because two of the four methyl groups in the asymmetric unit are undergoing fast tunnelling.

If the low temperature relaxation minimum for valine is generated by two methyl groups in the asymmetric unit, and the high temperature minimum by two $-NH_3$ groups, we might expect a ratio of relaxation constants in the region 1·2 to 1·4 as found in alanine, threonine and norleucine ; in fact it is 1·02 (table 1). The greater efficiency of the methyl rotors in this compound may perhaps be attributed to the modulation of the inter-methyl dipolar interaction, since both methyl groups in the molecule are attached to the same carbon atom and therefore in close proximity.

Support for this interpretation comes from the observation of Zaripov [8] of an additional relaxation minimum about 90 K, which may be attributed to the less hindered methyl groups. An unexpected feature of this lower minimum is that the value of $T_{1\ \mathrm{min}}$ is 2·2 times higher. Zaripov attributed this difference to longer inter-proton distances in the two methyl groups responsible for the lower temperature minimum ; they would however need to be about 14 per cent longer which seems rather unlikely. One alternative explanation is that the lower temperature minimum found by Zaripov is due to only one of the tunnelling methyl groups, and that one needs to go to still lower temperatures to find the effects of the fourth methyl group ; the four methyl groups in the asymmetric unit do after all have different crystallographic environments. Another alternative explanation is that the lower minimum is indeed due to the two tunnelling methyl groups, but that tunnelling reduces their relaxation efficiency ; there was some evidence of this in leucine. We should note too that since there are two molecules with different conformations in the asymmetric unit it is not unexpected that the relaxation data in figure 4 are fitted less well than for the other amino acids with just two independent relaxation processes. Finally it will be seen that the predicted values of T_n (table 2) for spectral narrowing are in very good agreement with observation (figure 6).

Further discussion of the relative values of E and τ_0 is deferred to paper III where a comparison is made for all the amino acids studied. We note however that for the methyl rotors values of E fall in the range 6·7 to 22·5 kJ/mole, while for the hydrogen-bonded $-NH_3$ groups the values of E fall in the higher range 32·5 to 51·7 kJ/mole.

The measurements of proton magnetic resonance spectra described in this paper were carried out at the University of Florida during the tenure of E.R.A. as Visiting Professor of Physics. We are most grateful to Professor T. A. Scott for providing facilities for this part of the work.

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