Conformations of Peptides in Solution by Nuclear Magnetic Resonance Spectroscopy. Part I. Application of Nuclear Magnetic Double Resonance Spectroscopy to the Determination of *cis*- and *trans*-Conformations of Peptide Bonds

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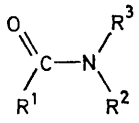
The 100 MHz ^{1}H n.m.r. spectra of some *N*-substituted amides and linear peptides have been measured. A small five-bond, long-range spin coupling has been shown to exist between groups antiperiplanar to each other across the amide and peptide bonds. In certain cases, ^5J(HH) has been observed between groups with a syncoplanar arrangement such that $^5J(HH)_{anti} > {}^5J(HH)_{syn}$. The observation of ^5J(HH) was used to assign the *cis*- and/or *trans*-conformations of peptide bonds in linear peptides containing *N*-methylated amino-acids. For unsubstituted peptide bonds the observation of ^5J(HH) between the C_α protons of adjacent amino-acids provides a direct method to confirm the presence of the *trans*-isomer of the peptide bond in linear or cyclic peptides in aqueous solutions.

THE amide bond and the peptide bonds have been shown to exist predominantly in the *trans*-conformation (I; $R^2 = H$) in most monosubstituted amides, peptides,



(I)

and proteins.[1] In cyclic amides and peptides the presence of the *cis*- or *trans*-isomer depends on the size of the ring.[2,3] Thus for small cyclic peptide molecules the constraints of ring formation demand a *cis*-conformation (I; $R^3 = H$) as found for the cyclic dipeptides,[4] *i.e.* substituted diketopiperazines. For hexa or higher cyclic peptides the *trans*-form of the peptide bond is found.[5]

Some peptide bonds, especially those found in certain cyclic peptide antibiotics, are formed from *N*-substituted amino-acids, *e.g.* sarcosine and *N*-methyl-L-valine. One property of such a bond found in disubstituted amides that distinguishes it from the monosubstituted amides is that the further substitution at the N atom promotes the stability of the *cis*-isomer at the expense of the *trans*-isomer.[6] This phenomenon has been extensively studied by n.m.r. spectroscopy, particularly in mono- and di-substituted amides. A number of n.m.r. studies have also been made on the physical properties of linear peptides containing *N*-methylated amino-acids in such molecules as *N*-acetylproline,[7] methyl *N*-acetylsarcosine,[8] t-Boc-Gly-L-Pro-OH and t-Boc-Gly-L-Pro-OBz,[4] *N*-acetyl-D-Ala-L-(NCH_3)-Ala and *N*-acetyl-(NCH_3)-Ala methyl ester.[9] In these dipeptide molecules *cis*- and *trans*-conformations of the *N*-methylated peptide bond exist in equilibrium in solution. In a series of cyclic sarcosine derivatives Dale and Titlestad[10] showed by n.m.r. studies that molecules exist exclusively in the *cis*-conformation for cyclodi- and tri-sarcosyl but that both *cis*- and *trans*-conformations exist in cyclotetra- and penta-sarcosyl and even in cyclo-octasarcosyl only one isomer (*cis*, *cis*, *trans*, *trans*, *cis*, *cis*, *trans*, *trans*) is observed. As there are some cyclic peptide antibiotics that contain *N*-methylated peptide bonds and as the conformation about each bond determines the overall shape of the molecule,[11] it is important to be able to distinguish which peptide bonds exist as the *cis*- or *trans*-isomer of such molecules in solution. We are investigating this problem by n.m.r. spectroscopy.

The terms *cis* and *trans* have been used to describe the conformations of the amide or peptide bond and also the relation of groups across the amide or peptide bond which show long-range spin coupling.[12-14] In order to avoid subsequent confusion, we use the terms *cis* and *trans* to describe the conformational isomers of amides and peptides and the terms *syn* and *anti* to indicate the relative positions of groups across the peptide bond which exhibit long-range spin coupling. The use of this nomenclature can be illustrated by reference to the general structure (I). In peptides and monosubstituted amides the *trans*-conformation has the amide proton (NH) *trans* to the amide carbonyl group, *i.e.* (I; $R^2 = H$). In *N*-methylated peptide bonds the *trans*-conformation refers to the isomer which has the peptide NCH_3 group *trans* to the peptide carbonyl group (I; $R^2 = CH_3$). In all amides and peptides proton spin coupling between

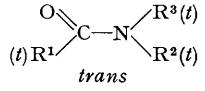
[1] (*a*) J. Donohue, *Proc. Nat. Acad. Sci. U.S.A.*, 1953, **39**, 470; (*b*) L. J. Bellamy, 'The Infra-red Spectra of Complex Molecules,' Methuen, London, 1958, 2nd edn., pp. 206—209.
[2] R. L. Jones, *Spectrochim. Acta*, 1967, *A*, **23**, 1745.
[3] J. Dale, *Angew. Chem. Internat. Edn.*, 1966, **5**, 1000.
[4] C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout, *J. Amer. Chem. Soc.*, 1970, **92**, 6191.
[5] F. A. Bovey, A. I. Brewster, D. J. Patel, A. E. Tonelli, and D. A. Torchia, *Accounts Chem. Res.*, 1972, 193, and references therein.
[6] L. A. La Planche and M. T. Rogers, *J. Amer. Chem Soc.*, 1964, **86**, 337.
[7] W. A. Thomas and M. K. Williams, *Chem. Comm.*, 1972, 788.
[8] A. L. Love, T. D. Alger, and R. K. Olsen, *J. Phys. Chem.*, 1972, **76**, 853.
[9] V. F. Bystrov, S. L. Portnova, V. I. Tsetlin, V. T. Ivanov, and Yu. A. Ovichinnikov, *Tetrahedron*, 1969, **25**, 493.
[10] J. Dale and K. Titlestad, *Chem. Comm.*, 1969, 656.
[11] C. H. Hassall and W. A. Thomas, *Chem. in Britain*, 1971, **7**, 145.
[12] E. W. Randall and J. D. Baldeschwieler, *J. Mol. Spectroscopy*, 1962, **8**, 365.
[13] D. G. de Kowalewski, *J. de Physique et le Radium*, 1962, **23**, 255.
[14] V. J. Kowalewski and D. G. de Kowalewski, *J. Chem. Phys.*, 1960, **32**, 1272.

groups which are antiperiplanar (I; R^1R^3) is designated J_{anti} and coupling between groups which are syncoplanar (I; R^1R^2) is designated J_{syn}.

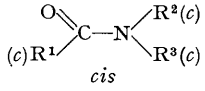
A number of n.m.r. methods have been used to distinguish between the *cis*- and *trans*-conformations of amide bonds for such molecules as dimethylformamide (DMF) and dimethylacetamide (DMA), *viz.* magnetic anisotropy,[15] aromatic solvent-induced chemical shifts,[16,17] nuclear Overhauser effect,[18] lanthanide-induced chemical shifts,[19] and proton spin coupling.[12,13] Only the latter method can be easily applied to determine the conformation of the amide bond when only one form (either *cis* or *trans*) is found.

In a study of *N*-methylformamide (NMF) and DMF Randall and Baldeschwieler [12] observed long-range coupling between the formyl proton and the methyl protons and they showed that the coupling for groups antiperiplanar to each other across the amide bond was greater than that for groups syncoplanar to each other, *i.e.* $^4J(HH)_{anti} > {}^4J(HH)_{syn}$. A five-bond, long-range proton spin coupling has also been observed in some disubstituted acetamides between groups antiperiplanar to each other across the amide bond.[13] Under certain solution conditions we have also observed a five-bond, long-range, proton spin coupling in the same disubstituted acetamides between groups syncoplanar to each other.[20] We have extended the measurements on amides to peptides and we will show that nuclear magnetic double resonance (n.m.d.r.) spectroscopy provides a general technique to determine the *trans*-conformation of peptides and the *cis*- and/or *trans*-conformation of *N*-substituted peptide bonds for oligopeptides in solution.

Long-range proton spin coupling constants in peptides



trans



cis

Compound	Solution	Conformation	R^1	R^2	R^3	Chemical shifts [δ (p.p.m.)] ±0·01 $R^1(c)$	$R^1(t)$	$R^2(c)$	$R^2(t)$	$R^3(c)$	$R^3(t)$	J/Hz (±0·05) [a] $R^1R^2(t)$ *syn*	$R^1R^3(t)$ *anti*	$R^1R^2(c)$ *anti*	$R^1R^3(c)$ *syn*
DMF	80% (v/v) D_2O	*cis*/*trans*	H	CH_3	CH_3	7·86	7·86	2·82	2·98	2·98	2·82	0·5 [b]	0·8 [b]	0·8 [b]	0·5 [b]
DMA	80% (v/v) D_2O	*cis*/*trans*	CH_3	CH_3	CH_3	2·07	2·07	2·87	3·02	3·02	2·87	0·1	0·5 [b]	0·5 [b]	0·1
N-Acetylsarcosine	D_2O, pD 7	*cis*/*trans*	CH_3	CH_3	CH_2	2·05	2·15	2·92	3·11	4·17	4·10	[c]	0·10	0·28	[c]
Glycyl-sarcosine	D_2O, pD 12·4	*cis*/*trans*	CH_2	CH_3	CH_2	3·36	3·52	2·94	2·99	3·88	3·92	[c]	0·15	0·4	[c]
Glycyl-*N*-methylvaline	D_2O, pD 7	*cis*/*trans*	CH_2	CH_3	CH	4·10	4·04	2·94	2·96	4·46	4·46	[c]	[c]	0·31	[c]
NMA	80% (v/v) D_2O	*trans*	CH_3	D	CH_3		1·98				2·70		0·5 [b]		
N-Acetylglycine	D_2O	*trans*	CH_3	D	CH_2		2·04				3·93		0·15		
N-Acetylalanine	D_2O	*trans*	CH_3	D	CH		2·00				4·24		0·15		
Glycyl-glycine	D_2O, pD 12·4	*trans*	CH_2	D	CH_2		3·40				3·82		0·20		
Glycyl-alanine	D_2O, pD 7	*trans*	CH_2	D	CH		3·77				4·10		0·40		
Glycyl-leucine	D_2O	*trans*	CH_2	D	CH		3·77				4·13		0·30		

[a] Values of spin coupling constants derived by measurements of line-widths at half-height of coupled and decoupled signals. [b] Values taken from peak separations of resolved multiplet signals. [c] No coupling between signals within experimental error.

EXPERIMENTAL

The samples of DMF, DMA, and *N*-methylacetamide (NMA) were obtained from B.D.H. and purified by fractional distillation under reduced pressure, dried and stored over molecule sieves. The samples of the dipeptides and the *N*-acylamino-acids were obtained from Sigma Chemical Co., and their purity checked by t.l.c. The sample of glycyl-*N*-methyl-L-valine was given by Dr. S. S. Danyluk, Argonne National Laboratory, Chicago.

Approximately 0·1M solutions of the dipeptides were made up in D_2O and measured in the zwitterion form. For some of the peptides the pD of the solution was adjusted to *ca.* 12 by NaOD since the proton signals were best separated at higher pD values. pD Was estimated from pH values measured with a glass electrode (pD = pH + 0·4). DMF, DMA, and NMA were dissolved in D_2O (20% v/v). Each solution was degassed by three successive applications of the freeze–thaw–pump method and sealed in an n.m.r. tube under vacuum with the appropriate internal reference sodium [2,2,3,3-$^2H_4$]-3-trimethylsilylpropionate (TSP) for D_2O solutions.

The n.m.r. spectra were recorded on a JEOL MH-100 spectrometer operating at a probe temperature of *ca.* 300 K. Measurements of line-widths were made of five successive scans of the coupled and decoupled spectra observed at a 54 Hz sweep width.

The relevant values of chemical shifts and spin-coupling constants are listed in the Table. The $^5J(HH)$ values for the amides were measured directly from line separations of resolved multiplets. The long-range coupling constants of the peptides are too small to observe directly from the splitting patterns of the various signals with the resolution available and so they have been deduced from line-widths of the coupled and decoupled signals. It is expected that measurements of line-widths at half-height of signals leads to erroneous values of derived spin coupling constants with such small values as 0·2—0·3 Hz found for these molecules; thus, the J values quoted in the Table are only approximate values. The correct J values can only be determined by complete line-shape analysis. However, in this work, it is the observation of long-range spin coupling and not the absolute values of coupling constants that is necessary to determine the conformation of the molecules.

RESULTS AND DISCUSSION

A. N-*Substituted Peptide Bonds.*—In a substantial review [2] on the application of n.m.r. to the study of amides, the use of n.m.d.r. in distinguishing between the *cis*- and *trans*-forms of the substituted amide bond in

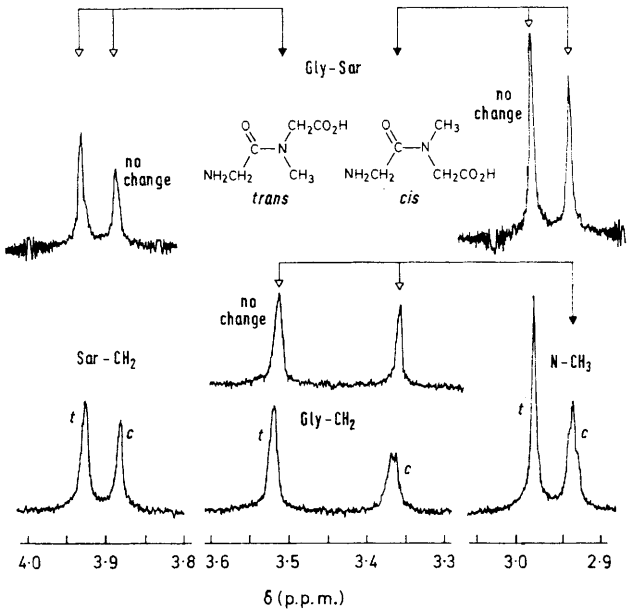
[15] H. Paulsen and K. Todt, *Angew. Chem. Internat. Edn.*, 1966, **5**, 899.
[16] J. V. Hatton and R. E. Richards, *Mol. Phys.*, 1960, **3**, 253.
[17] D. L. Hooper and R. Kaiser, *Canad. J. Chem.*, 1965, **43**, 2363.
[18] F. A. L. Anet and A. J. R. Bourn, *J. Amer. Chem. Soc.*, 1965, **87**, 5250.
[19] C. Beauté, Z. W. Wolkowski, and N. Thoai, *Chem. Comm.*, 1971, 700.
[20] D. B. Davies and Md. Abu Khaled, to be published.

DMF and DMA when they both exist in solution was shown. In particular, the four-bond, long-range coupling has been observed in DMF [12] and other substituted formamides [13] such that for groups *anti* to each other across the amide bond a coupling of *ca.* 0·8 Hz is observed but for groups *syn* to each other a coupling of 0·4—0·5 Hz has been observed, *i.e.* $^4J(HH)_{anti} > {}^4J(HH)_{syn}$. Our measurements for DMF in a D_2O solution (I; $R^1 = H$, $R^2 = R^3 = CH_3$) show that under conditions of high resolution the upfield *N*-methyl signal (δ 2·81) consists of a doublet with peak separations of 0·8 Hz whilst the downfield *N*-methyl signal (δ 2·98) consists of a doublet with peak separation of 0·5 Hz. In a double resonance experiment each doublet collapsed to a singlet when the formyl proton was irradiated. The two *N*-methyl peaks have been previously assigned [21] such that the upfield signal results from the methyl group syncoplanar with respect to the carbonyl group [Table; $R^2(c) = R^3(t) = CH_3$] and the downfield signal results from the methyl group antiperiplanar to the carbonyl group (Table; $R^2(t) = R^3(c) = CH_3$). Thus we observe for groups antiperiplanar to each other across the amide bond in DMF that $^4J(HH) = 0·8$ Hz and for groups syncoplanar to each other $^4J(HH) = 0·5$ Hz for DMF in D_2O solutions, *i.e.* $^4J(HH)_{anti} > {}^4J(HH)_{syn}$.

A five-bond, long-range, spin coupling has been observed previously in DMA [13] (I; $R^1 = R^2 = R^3 = CH_3$) for groups which are antiperiplanar to each other across the amide bond. Our measurements for DMA in D_2O solutions show one signal for the acetyl methyl protons (δ 2·07) and two signals for the *N*-methyl group for the *cis*- and *trans*-isomers. Under conditions of high resolution at 54 Hz sweep width it is observed that the upfield *N*-methyl signal (δ 2·87) exists as a quartet with peak separations of 0·5 Hz, whilst the downfield *N*-methyl signal (δ 3·02) is observed as a broad singlet. In a double resonance experiment irradiation of the acetyl methyl signal decouples the small long-range spin coupling so the quartet collapses to a singlet and, at the same time, the singlet *N*-methyl signal sharpens considerably so that both the *N*-methyl signals appear equally intense and with equal line-width. The *N*-methyl signals of DMA have been assigned previously by various methods [21] such that the upfield signal results from the methyl group syncoplanar with respect to the carbonyl group and the downfield signal results from the methyl signal antiperiplanar to the carbonyl group for DMA which exists as an equilibrium mixture of the *cis*- and *trans*-isomers in solution.

We observe for methyl groups which are antiperiplanar to each other in DMA that $^5J(HH)_{anti} = 0·5$ Hz, which was determined from the resolved quartet *N*-methyl signal [Table; $R^2(c) = R^3(t) = CH_3$]. The coupling for groups which are syncoplanar can be observed from the sharpening of the *N*-methyl signal [Table; $R^2(t) = R^3(c) = CH_3$] but the value can only be estimated by line-width measurements. It should be noted that in DMA the latter *N*-methyl signal which is syncoplanar to the carbonyl should appear as a quartet due to coupling with three magnetically equivalent acetyl methyl protons. The observed difference in line-width at half signal height between the coupled and decoupled signal of 0·3 Hz is divided by 3 to give the value of $^5J(HH)_{syn}$ of 0·1 Hz in the Table. We observe for dimethylacetamide that $^5J(HH)_{anti} > {}^5J(HH)_{syn}$. It can be seen from the Table that the five-bond, long-range coupling between groups *syn* or *anti* to each other across the amide bond is less than the corresponding four-bond coupling in DMF.

Having established the $^5J(HH)$ spin-coupling behaviour for groups which are *syn* or *anti* to each other across an amide bond, we are now in a position to investigate the possibility of long-range coupling between the *N*-methyl protons and the C_α protons of the preceding



100 MHz ^1H n.m.r. spectrum of glycyl-sarcosine (Gly-Sar) in D_2O at pD 12·4 showing results of decoupling experiments. Horizontal lines connect coupled signals. The filled triangles represent the position of the second irradiating field and the open triangles represent the position of the observing field in a double resonance experiment

residue for an *N*-methylated peptide bond in either the *cis*- or *trans*-conformation.

N-Substituted Peptides.—We have mentioned the previous n.m.r. studies [4,7-9] of *N*-methylated peptide bonds in which *cis*- and *trans*-isomers of the peptide bond exist in equilibrium in solution. We are examining the physical properties of a number of *N*-substituted linear peptides by ^1H n.m.r. spectroscopy. The examples chosen for the present study are *N*-acetylsarcosine, glycyl-sarcosine, and glycyl-*N*-methyl-L-valine. We will illustrate the use of spin-decoupling experiments to determine the preferred conformation of these molecules by analysing the spectrum of Gly-Sar measured in D_2O solution. The 100 MHz spectrum of Gly-Sar in D_2O at pD 12·4 measured at 54 Hz sweep width is shown in the Figure to consist of three sets of peaks at δ 3·90,

[21] W. E. Stewart and T. H. Siddall, *Chem. Rev.*, 1970, **70**, 517.

3·45, and 2·96 which correspond to the Sar-CH_2, Gly-CH_2, and Sar-CH_3 signals respectively. Each set of protons gives rise to two peaks of unequal intensity which indicates that the rotation about the peptide bond is sufficiently slow on the n.m.r. time scale at ambient temperatures for observation of both *cis*- and *trans*-isomers. The restricted rotation about the peptide bond was confirmed by heating the D_2O solution with Gly-Sar in the zwitterion form when the three sets of peaks collapsed to three singlets at temperatures >360 K. The chemical shifts of the two methylene peaks were assigned by following the titration of Gly-Sar by n.m.r. and noting that the methylene signal which moved upfield at higher pD values could be assigned to the Gly-CH_2 signal as observed previously for various glycine oligomers by Nakamura and Jardetzky.[22]

The nomenclature for the proton signals in different environments is shown by the *cis*- and *trans*-isomeric structures in the Figure and Table. For the molecule in the *cis*-conformation the Sar-CH_3 group is *cis* to the carbonyl group and so we have labelled both methylene groups for this isomer by the *cis*-convention, *i.e.* Gly-$CH_2(c)$ and Sar-$CH_2(c)$. On the other hand, for the *trans*-isomer the groups are designated Gly-$CH_2(t)$, Sar-$CH_2(t)$, and Sar-$CH_3(t)$.

By analogy with the peak assignments for the *N*-substituted amides, it is expected that the upfield *N*-methyl peak corresponds to the signal for the *cis*-isomers, *i.e.* $CH_3(c)$ as in the Figure. Similar behaviour was found for *N*-CBZ-Gly-Sar, which is soluble in [$^2H_6$]acetone and confirmation of the assignment of the *cis*- and *trans*-*N*-methyl signals was made by another established method, *i.e.* aromatic solvent-induced chemical shifts. The intensity of the $CH_3(c)$ signal is about one-half that of the $CH_3(t)$ signal and this intensity difference serves to assign the *cis*- and *trans*-peaks for the two sets of methylene protons as shown in the Figure. The expansion of all these signals to 54 Hz sweep width shows that the $CH_3(c)$ peak is broader than the $CH_3(t)$ peak, the Sar-$CH_2(t)$ signal is broader than the Sar-$CH_2(c)$ signal, and the Gly-$CH_2(c)$ signal is broader than the Gly-$CH_2(t)$ signal. The differences in line-widths of the various signals can be explained in terms of long-range coupling which can be demonstrated by the various spin-decoupling experiments summarised in the Figure. Thus irradiation of the Gly-$CH_2(c)$ signal sharpens the $NCH_3(c)$ signal but has no effect on the line-width of the Sar-$CH_2(c)$ signal within the measuring error of five successive decoupling experiments. The ^5J(HH) coupling was confirmed and checked by irradiation of the N-$CH_3(c)$ signal in which there is no effect on the Gly-$CH_2(t)$ signal within experimental error but the Gly-$CH_2(c)$ signal sharpens, as shown in the Figure. These experiments show that for the *cis*-form of Gly-Sar there is coupling between the Sar-NCH_3 protons and the Gly-CH_2 protons which are antiperiplanar to each other across the peptide bond but that there is no coupling between the Gly-CH_2 and Sar-CH_2 protons which are syncoplanar to each other for this conformation, *i.e.* $^5J(HH)_{anti} > {}^5J(HH)_{syn}$.

It is expected that coupling should also be observed between the Gly-CH_2 and Sar-CH_2 signals in the *trans*-isomer of Gly-Sar, as these groups are now *anti* to each other across the peptide bond. We can demonstrate this coupling by irradiation of the Gly-$CH_2(t)$ signal and observing a decrease in the line-width of the Sar-$CH_2(t)$ signal, whereas there is no effect within experimental error on the Sar-$CH_2(c)$ signal.

The two other peptides containing *N*-methylated bonds measured in this work, *i.e.* *N*-acetylsarcosine and glycyl-*N*-methyl-L-valine show spin coupling characteristics similar to those found for glycyl-sarcosine. The spectrum of *N*-acetylsarcosine measured in a D_2O solution in this study shows the same features arising from the *cis*- and *trans*-isomers as found previously for *N*-acetylsarcosine methyl ester.[8] The assignment of the spectrum was checked by spin-decoupling experiments and the ^5J(HH) values measured. It can be seen from the Table that, for peptides, groups antiperiplanar to each other are coupled with $^5J(HH)_{anti}$ *ca.* 0·2—0·4 Hz whereas groups syncoplanar to each other are not coupled within experimental error.

The decoupling experiments are consistent with the assignment of the spectra of these molecules from chemical shift values. We have found that proton spin-decoupling can also be used for assigning the *cis*- and *trans*-isomers of *N*-methylated peptide bonds as well as with *N*-substituted amides. Thus the observation of long-range coupling has a diagnostic role in determining the *cis*- and/or *trans*-isomers of a peptide bond. For example, in a linear or cyclic peptide containing an *N*-methylated peptide bond only one conformation may be found in solution. This conformation would give rise to a single n.m.r. peak for the *N*-methyl group of either the *cis*- or *trans*-isomer. Assignment of either the *cis*- or *trans*-conformation from the chemical shift of one peak would be difficult, whereas observation of long-range coupling as shown in these model compounds could distinguish between the two isomeric forms. Thus, if long-range coupling can be observed between the *N*-methyl group and the C_α protons of the preceding residue in an *N*-methylated peptide, the peptide can be assigned the *cis*-conformation. On the other hand, if long-range coupling is observed between the C_α proton of one residue and the C_α proton of the preceding residue, the *trans*-conformation can be assigned for the *N*-methylated peptide. These conditions also apply to the unsubstituted peptide bond in either the *cis*- or *trans*-conformation. We will examine the applicability of ^5J(HH) between groups antiperiplanar to each other across a peptide bond.

B. *The Peptide Bond.*—Many *X*-ray crystallographic studies have shown that the peptide bond exists in the *trans*-form in linear peptides and proteins and also in many cyclic peptides containing six or more residues. Other methods such as i.r., o.r.d., and c.d. have shown that the peptide bond exists in the *trans*-form in solution,

[22] A. Nakamura and O. Jardetzky, *Biochemistry*, 1968, **7**, 1226.

though exceptions are provided by the cyclodipeptides and the *N*-methylated peptide bond discussed in the previous section.

Although n.m.r. spectroscopy is used extensively to determine the configuration and conformation of molecules in solution, the methods found useful in substituted amides and peptides, *e.g.* chemical shifts, aromatic solvent-induced shifts, nuclear Overhauser experiments, and lanthanide-induced shifts, cannot be readily applied to determine the *trans*-conformation of peptides in solution. We have shown that long-range proton spin coupling can be used to determine groups which are antiperiplanar to each other across *N*-methylated peptide bonds and we will now examine the conditions under which this phenomenon can be applied to groups *anti* to each other across peptide bonds.

The phenomenon of long-range coupling has been observed previously in monosubstituted amides by Kowalewski [13,14] who showed that for *N*-methylacetamide $^5J(\mathrm{HH})_{anti}$ is about 0·45—0·5 Hz, whereas $^5J(\mathrm{HH})_{syn}$ is zero. We have observed $^5J(\mathrm{HH})$ for NMA in D_2O solutions where the presence of *cis*- and *trans*-isomers have been observed previously.[23] We found that $^5J(\mathrm{HH})_{anti}$ for NMA increases from a value of 0·28 for a 90% (v/v) solution in D_2O to 0·55 Hz for a 10% (v/v) D_2O solution. The latter coupling was determined directly from the observed quartet splitting patterns of the methyl signals and the value is similar to the 0·6 Hz observed previously for NMA in a D_2O solution.[23] Having established the presence of long-range coupling between groups *anti* to each other across the amide bond, we examined the same phenomenon for the peptide bond found in *N*-acetylglycine, *N*-acetylalanine, glycylglycine, glycyl-alanine, and glycyl-leucine. We will illustrate this phenomenon using glycyl-glycine.

Glycyl-glycine, Gly(1)-Gly(2).—We have labelled the glycine at the amino-end of the dipeptide as Gly(1) and the glycine residue at the carboxylate end as Gly(2). In the *trans*-conformation of the peptide bond the two glycine methylene groups are antiperiplanar to one another. The 100 MHz n.m.r. spectrum of Gly-Gly in D_2O solutions at pD 12·4 shows that the two glycine methylene signals are well separated by 0·42 p.p.m. The upfield methylene signal can be assigned to the Gly(1) residue and the downfield methylene can be assigned to the Gly(2) residue at high pD values.[22,24] Irradiation of the Gly(2) methylene signal sharpens the Gly(1) signal and *vice versa*, showing that long-range coupling can be observed between them. By comparison with the decoupling experiments for molecules containing *N*-methylated peptide bonds, the observation of a $^5J(\mathrm{HH})$ in peptides suggests that the two methylene groups are *anti* to each other, *i.e.* the peptide bond exists in the *trans*-conformation in solution. Similar behaviour was found for *N*-acetylglycine, *N*-acetylalanine, glycyl-alanine, and glycyl-leucine as shown in the Table. Thus n.m.d.r. spectroscopy provides a method for the direct confirmation of the *trans*-form of the peptide bond for peptides in aqueous solution.

[3/816 *Received*, 16*th April*, 1973]

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