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CONFORMATIONAL STUDIES OF PEPTIDE SYSTEMS

THE ROTATIONAL STATES OF THE NH—CH FRAGMENT OF ALANINE DIPEPTIDES BY NUCLEAR MAGNETIC RESONANCE[1]

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Abstract—The IR and NMR-H[1] spectra of alanine dipeptides and their N-Me derivatives have been investigated. Measurement of the integral intensities of the N—H stretching vibrations in dilute solutions of CCl_4 and of a (1:9) $CHCl_3$ + CCl_4 mixture showed that in these solvents approximately 70% of the alanine dipeptide molecules are in an intramolecular hydrogen-bonded folded form. It was found from analysis of the vicinal proton spin–spin coupling constant of the CONH—CH fragment that the stable conformer with respect to this bond is that with *cis*-arrangement of the NH and CH hydrogens. The conformation of the 7-membered hydrogen-bonded ring of the dipeptides has been elucidated. An empirically found stereochemical dependence of the constant $^3J_{NH-CH}$ upon the dihedral angle θ of the fragment has served as basis for discussing the possible conformations of the extended form of the dipeptides in polar (including aqueous) solvents.

I. INTRODUCTION

THE interest of peptide chemists is at present being focused more and more on the finer points of the spatial structure of peptide systems.[2a–e]

This is quite natural, for while it is certain that such structural details play an important part in biological activity of peptides and proteins there are as yet practically no reliable data on the conformational states of these substances in solution. The difficulty of this problem is due to the large variety of factors that can influence the spatial structure of peptide chains. Such, for instance, are steric and polar interactions of neighbouring groups, hydrogen-bonding, solvent–solute interaction including hydrophobic interactions, etc.

Theoretical calculations of the energy parameters for the conformational states of polypeptides carried out by a number of authors[3a–k] have indicated the possible existence of preferred orientations of the peptide chain. Such conclusions, however plausible, still require experimental verification.

One of the approaches to the problem of the geometrical structure of the peptide chains in solutions is determination of the conformational states of simple peptides by physicochemical means. The correlations obtained could possibly serve as a key to the relationships governing the basic chain structure of the more complex, naturally occurring polypeptides and proteins. Consequently, we have made an IR and NMR study of stereoisomeric α-alanine dipeptides in solution.

II. RESULTS

The present paper reports the results of the IR and NMR studies of methyl esters of a number of alanine dipeptides and their N-Me derivatives (Table 1).

TABLE 1

(7) (6) (5) (4) (3) (2) (1)
R′—CO—N—CH—CO—N—CH—COOCH$_3$
R″ CH$_3$ R‴ CH$_3$

Dipeptide	R′	R″	R‴
I, DD	$C_6H_5CH_2O$—	H	H
II, DL	$C_6H_5CH_2O$—	H	H
III, DD	CH_3	H	H
IV, DL	CH_3	H	H
V, DD	$C_6H_5CH_2O$—	H	CH_3
VI, DL	$C_6H_5CH_2O$—	H	CH_3
VII, DD	CH_3	CH_3	H
VIII, DL	CH_3	CH_3	H
IX, DD	CH_3	H	CH_3
X, DL	CH_3	H	CH_3

(a) *IR spectra*

Quantitative determination of the folded form in alanine dipeptides. It has been repeatedly demonstrated that simple molecules containing amide and peptide bonds undergo intermolecular association by NH...O=C hydrogen-bonding.[2d, 4, 5] Certain fragments of the peptide chain manifest a tendency to fold, particularly when the resultant structure is stabilized by intramolecular hydrogen-bonds.[2d, 6] An attempt has been made to estimate the energy of such a bond in molecules with two peptide bonds by determining the temperature dependence of the intensity of the NH stretching band.[6] It should, however, be mentioned that the peak intensities do not allow a sufficiently accurate estimate of the fraction of folded form.[4]

In order to determine this and also to obtain more accurate thermodynamic data on the folded-extended form equilibria we measured the integral IR intensities for dilute CCl_4 solutions of the dipeptides. The concentration (3 to 5) . 10^{-4} moles/l., was sufficiently low to eliminate intermolecular association between the peptide molecules. It was found that neither the $N_{(3)}$-methylated derivatives (V, VI, IX and X) nor N-acetyl-D-alanine methyl ester form intramolecular hydrogen-bonds. From this it followed that the intramolecular hydrogen-bond in the folded form of the molecules I–IV and VII–VIII is between $N_{(3)}H$ and $C_{(7)}=O$, giving rise to a 7-membered ring. This is in full accord with the results of Mizushima for systems with two peptide bonds.[2d, 6]

Based on a comparison of the integral stretching band intensities of the free NH groups ($D_{NH\ free}$) in the IR spectra of the alanine dipeptides (III, IV) and the $N_{(3)}$-methylated compounds IX and X, we calculated the relative amount n of the intramolecular hydrogen-bonded molecules. The results are presented in Table 4 of Ref. 1*b* which shows that the fraction of the hydrogen-bond stabilized folded forms amounts to about 70%. A study of the temperature dependence of the integral free NH band intensity in the case of N-acetyl-D-alanyl-L-alanine methyl ester (IV) made it possible to determine the entropy ΔS and enthalpy ΔH differences between the folded and extended forms in CCl_4 solution.* These parameters were found to be of

* Thermodynamic data represents the average value for all possible forms with intramolecular hydrogen-bond

lower value for the folded form: $\Delta S = -(9{\cdot}8 \pm 0{\cdot}7)$ e.u. and $\Delta H = -(3{\cdot}1 \pm 0{\cdot}2)$ kcal/mole. The decrease in entropy of the folded form is apparently due to its greater hindrance to internal rotation than the extended form. At least in part, stabilization of the folded form is due to intramolecular hydrogen-bonding. For more detailed discussion of the experimental procedure, the method for calculating the fraction of folded form and the equilibrium thermodynamic parameters, and also for synthesis of the compounds.[1b]

In order to interconnect the IR and NMR data the gap between the conditions for obtaining both types of spectra should be made as narrow as possible. Especially does this pertain to the concentration of the solutions. Since it was impossible to increase the concentration because of the low solubility of the dipeptides in CCl_4, IR spectra were taken of 0·03 to 0·01 moles/l solutions of I, III, V, VI and IX in a binary $CCl_4 + CHCl_3$ mixture with minimal (9:1) amount of chloroform. The data obtained are given in Table 2. The IR spectra of I, and III in the binary solvent, as in

TABLE 2. INTEGRAL INTENSITIES OF THE HIGH FREQUENCY (FREE) BAND ν_{NH} OF THE ALANINE DIPEPTIDES AND THE FRACTION OF THE FOLDED FORM n IN (1:9) $CHCl_3 + CCl_4$ MIXTURE

Compound	Concentration in moles/l	D 10^{-4} l./mol. cm^2	Fraction of the free NH groups in %	Fraction of the bonded NH groups in %	Fraction of the folded form n
I	0·0308	1·34	69	31	0·64
III	0·0300	0·95	49	51	0·75
V	0·0150	0·99	100	—	—
VI	0·0150	1·02	100	—	—
IX	0·0149	0·86	89	11	—
	0·0298	0·71	73	27	—

CCl_4 alone, exhibit two NH stretching frequencies. The low frequency band (ν_{NH} = 3340 cm^{-1}) being due to intra- and intermolecular hydrogen bonded NH groups of the type NH...O=C and the high frequency band (ν_{NH} = 3420 cm^{-1}) to groups not forming such bonds. However, the latter can take part in the formation of hydrogen-bonds with chloroform, which is apparently the cause for the 20 cm^{-1} shift to lower wavelengths of their adsorption as compared with the CCl_4 solutions. In the IR spectrum of IX in the 0·03 and 0·015 moles/l. binary solvent solutions one also can observe the appearance of a broad low frequency band near 3310 cm^{-1} which corresponds in this case to 27 and 11% intermolecular hydrogen-bonding (see Table 2).

The IR spectra of compounds V and VI display only the high frequency NH band, which shows that no self-association occurs at concentrations of 0·015 moles/l. The integral intensity of this band equals within the limits of experimental error ($\pm$0·05) the intensity D_0 of the free NH group for solutions of V, IX and X in CCl_4 (cf. Table 2 and Table 4 in Ref 1*b*). Taking D_0 to be $0{\cdot}97 \cdot 10^4$ l. . mole^{-1} . cm^{-2} one can calculate the self-association for solutions of IX and also the fraction of NH groups not participating in the NH...O=C hydrogen-bond for solutions of I and

III (Table 2). The lower integral intensity of the high frequency (3420 cm^{-1}) band in the IR spectra of IX as compared with V and VI bears evidence of self-association of the N-acetyl dipeptide at the given concentration (Table 2).

Assuming the $N_{(6)}$-acetylated dipeptides III and IX to have the same degree of self-association at equal concentrations, the fraction of molecules of the intramolecular hydrogen-bonded folded form can be estimated. The $C_{(7)}$-benzyloxycarbonyl dipeptides V and VI do not undergo self-association so that the folded form fraction for I can be calculated directly[1b] from the equation $n = 2\text{-}D_{NH\ free}/D_0$. The results of the calculation, summarized in Table 2, show that the fraction of the folded form is approximately the same in the binary solvent as in the CCl_4 solution.

The IR data then served as the starting point for the NMR treatment of the NH—CH rotational states of the alanine dipeptides.

(b) *Proton magnetic resonance (NMR-H[1]) spectra*

The NMR-H^1 spectra of representatives of all the three types of the compounds investigated (benzyloxycarbonyl derivatives, N-acetylated derivatives and N-acetyl-N-methyl derivatives of alanine dipeptides) are shown in Figs 1, 2 and 3, respectively.

For the benzyloxycarbonyl derivatives (I and II) and the N-methylated compounds (V–X) assignments of the signals are made without difficulty. The $N_{(6)}H$ signal of the benzyloxycarbonyl derivatives of the DD and DL isomers (I and II) can be readily distinguished from the considerably lower field $N_{(3)}H$ signal. (Fig. 1). The increased screening of the urethane ROCO—NH—R′ proton as compared with the RCONHR can be seen from spectra No. 85 and 319 in[7] and also.[8]

The increased screening of the urethane proton is apparently due to the effect of the side chain oxygen upon the electron density distribution on the nitrogen. Assignment of the $N_{(3)}H$ signal in the spectra of I and II follows also from a comparison with the spectra of the N-acetyl derivatives of the alanine dipeptides III, IV (see below).

The application of proton–proton double resonance[9] (NMR H^1—H^1) permitted

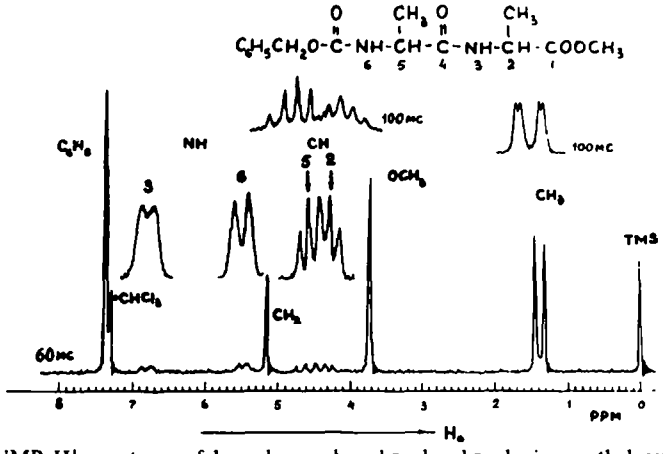


FIG. 1 NMR-H^1 spectrum of benzyloxycarbonyl-D-alanyl-D-alanine methyl ester (I) in $CDCl_3$ (0·18 moles/l.) at 60 Mc/s. In the upper part of the figure there is a fragment of the spectrum at 100 Mc/s.

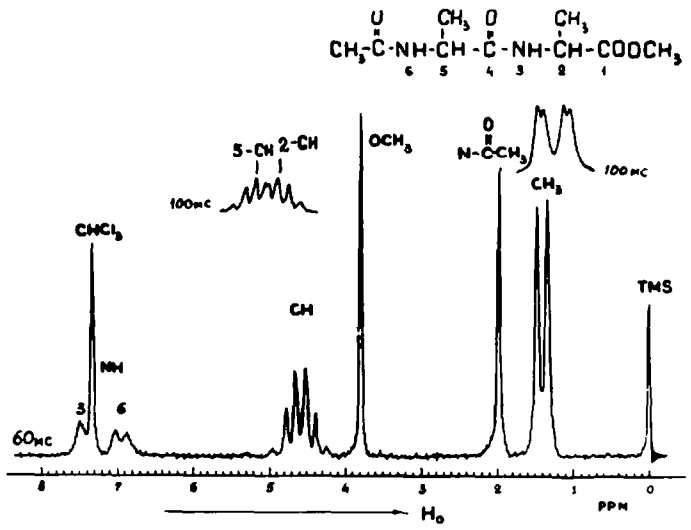


FIG. 2 NMR-H[1] spectrum of N-acetyl-D-alanyl-D-alanine methyl ester (III) in $CDCl_3$ (0·15 moles/l.) at 60 Mc/s. In the upper part of the figure there is a fragment of the spectrum at 100 Mc/s

assignment of the CH methines in the spectra of the benzyloxycarbonyl derivatives (I and II). For instance irradiation by a radio frequency field of the upfield reasonating CH proton causes collapse of the lower field NH doublet. Hence the irradiated proton belongs to the $C_{(2)}H$ group. In a similar way it was shown that the down-field signal is due to the $C_{(5)}H$ proton. This was confirmed by comparing the respective line widths in the NMR-H[1] spectra of the compounds I and II at 100 Mc/s. The $N_{(3)}$—H doublet in the lower field has wider components than that of the $N_{(6)}H$ doublet (Fig. 1). Hence the broadened CH multiplet must belong to $C_{(2)}H$. This multiplet is at somewhat higher field than the narrow line one assigned to $C_{(5)}H$. It appears that the different widths of the urethane and amide proton signals is the result of the different relaxation rates of the corresponding protons, due either to quadrupole relaxation of the N^{14} nitrogen or to hydrogen exchange.[10]

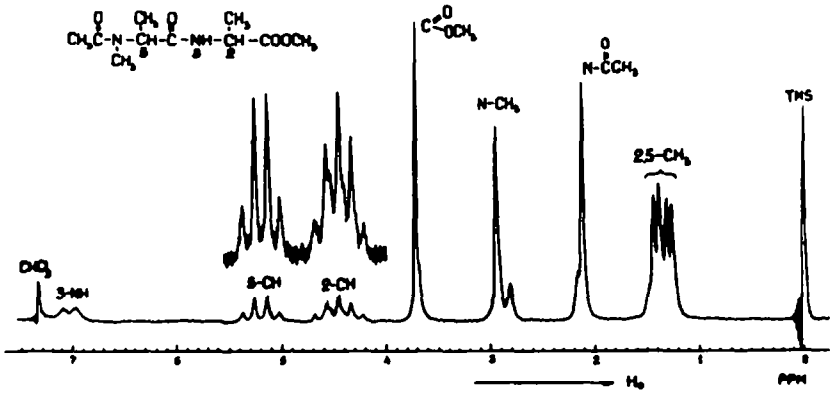


FIG. 3 NMR-H[1] spectrum of N-acetyl-D-N-methylalanyl-D-alanine methyl ester (VII) in $CDCl_3$ (0·20 moles/l.) at 60 Mc/s.

Assignment of the signals in the spectra of the N-acetyl-alanine dipeptides proved to be much more difficult because of the close values for both NH shifts (Fig. 2). For assignment of the Me proton signals a comparison was made of the spectra for the aqueous (D_2O) solutions of the DD and DL isomers (III, IV) with and without addition of NaOD. As is well known saponification of the ester groups of the dipeptides occurs in alkaline medium with formation of the corresponding salts:[11]

$$\underset{\text{III}}{\text{AcAlaAlaOCH}_3} \longrightarrow \underset{\text{IIIa}}{\text{AcAlaAlaO}^-\text{Na}^+} + \text{CH}_3\text{OD}$$

As a result the $C_{(2)}$-Me signal should be displaced whereas the $C_{(5)}$-Me would be expected to retain the former chemical shift. Fig. 4a shows two overlapping doublets in the Me region of the NMR-H^1 spectrum of the DD isomer (III) in D_2O. Fig. 4b

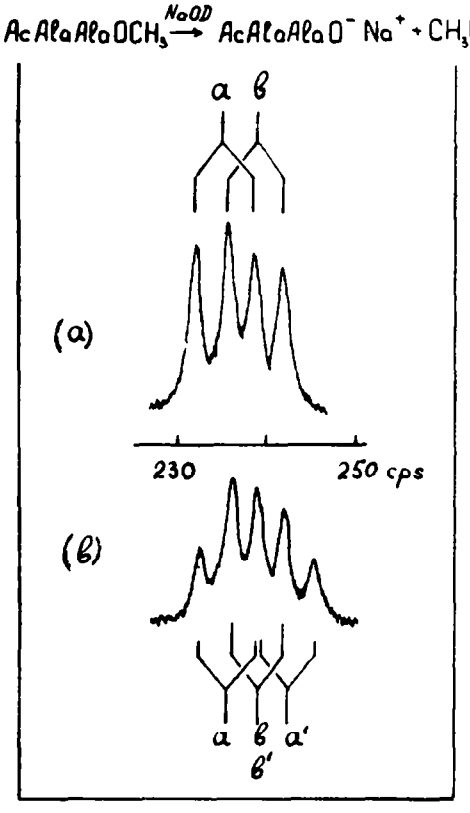


FIG. 4 (a) 100 Mc/s NMR-H^1 spectral region of the Me signals for N-acetyl-D-alanyl-D-alanine methyl ester (III) in D_2O (0·15 moles/l.). (b) The same with addition of NaOD The frequency scale in c/s is with reference to dioxan as internal standard

shows the same region after addition of a small amount of NaOD. From the intensities of the separate components it can be seen that saponification must have occurred to the extent of 50%, i.e. the sample now contains compounds III and IIIa in an approximately 1:1 ratio.

From a comparison of the two spectra in Fig. 4 it follows that the signal *a'* of the methyls undergoes an upfield shift of 0·06 ppm in the case of the sodium salt. As was

to be expected, the chemical shift of the signal *b'* was not noticeably displaced: the signal *b* and b' coinciding (Fig. 4b). The observed effect is in conformity with the results obtained in a study of the dependence of the chemical shifts of non-protected di- and tripeptides upon the pD of aqueous solutions.[12]

Since the mobile signal *a* can now be ascribed to the group $C_{(2)}$—CH_3, the second signal *b*, is consequently due to $C_{(5)}$—CH_3.

The Me proton signals for acetone solutions of the alanine dipeptides were assigned by observing the spectral changes on gradual addition of acetone to the D_2O solution. It was found that the signals did not interchange their higher and lower field positions, the $C_{(2)}$—CH_3 signal being always at lower field than the $C_{(5)}$—CH_3 signal.

The NMR-H^1 spectrum of a solution of N-acetyl-D-alanyl-D-alanine (III) in $(CD_3)_2$ CO at $-10°$ is presented in Fig. 5. The temperature was lowered to retard

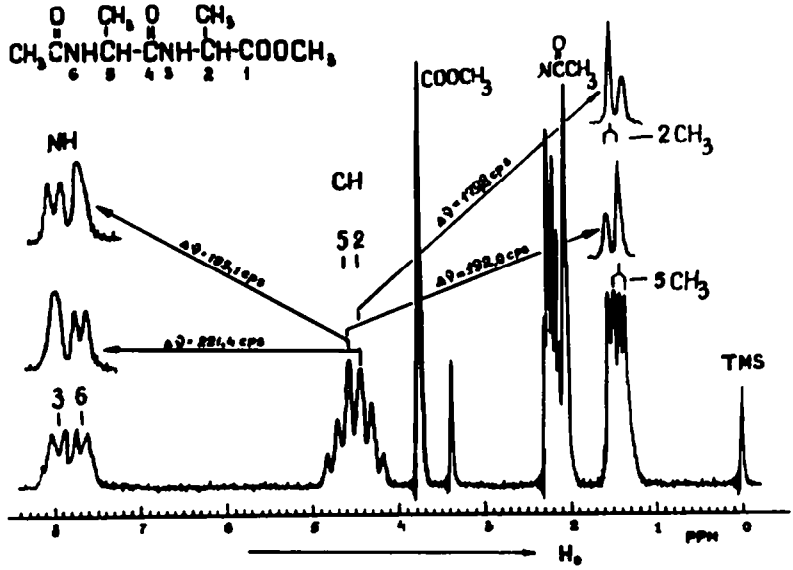


FIG. 5 Double resonance NMR H^1—H^1 spectrum of N-acetyl-D-alanyl-D-alanine methyl ester (III) in $(CD_3)_2CO$ (0·14 moles/l.) at 60 Mc/s. The lower part of the figures shows the NMR-H^1 spectrum, the upper part, the double resonance experiments The signals at 2·2 and 3·4 are due to molecules of (CHD_2) $(CD_3)CO$ and H_2O, respectively, present in the solution.

hydrogen exchange between the NH protons and water molecules present as impurity in the acetone. Assignment of the CH and NH signals was achieved by means of total double reasonance. As a result of experiments on CH_3—{CH} it was established that the lower field methine signal is due to the $C_{(5)}H$, and the higher field signal to $C_{(2)}H$. From this and experiments on NH—{CH} it follows that the $N_{(3)}H$ doublet is at lower field than the $N_{(6)}H$ doublet.

Assignment of the NMR-H^1 signals of III in $CDCl_3$ was made by gradual addition of the latter to the acetone solution. In a similar way assignments were made for the signals of N-acetyl-D-alanyl-L-alanine methyl ester (IV). The proton chemical shifts for the respective compounds are given in Table 3.

The NH resonances depend to a marked extent on the nature of the solvent, the

TABLE 3. CHEMICAL SHIFTS OF THE PROTONS OF ALANINE DIPEPTIDES AND THEIR DERIVATIVES (IN PPM RELATIVE TO TMS)

Compound	Solvent and concentration in moles/l.	$C_{(2)}CH_3$	$C_{(5)}CH_3$	$C_{(2)}H$	$C_{(5)}H$	$N_{(3)}H$	$N_{(6)}H$	$COCH_3$ *trans*	CH_2	$N—CH_3$ *trans*	$N-CH_3$ *cis*	$—OCH_3$ *trans*
I	$CDCl_3$, 0·25	1·36	1·38	4·33	4·55	6·96	5·78	—	5·10	—	—	3·73
II	$CDCl_3$, 0·25	1·38	1·38	4·32	4·56	6·97	5·72	—	5·12	—	—	3·73
III	$CDCl_3$, 0·25	1·39	1·41	4·51	4·64	7·41	6·95	2·01	—	—	—	3·75
	$CDCl_3 + (CH_3)_2SO$ (10:1), 0·17	1·32	1·36	4·46	4·53	7·75	7·39	1·97	—	—	—	3·70
	H_2O, ~0·1	1·62	1·65	—	—	7·89	7·65	2·25	—	—	—	3·60
IV	$CDCl_3 + (CH_3)_2SO$ (10:1), 0·17	1·30	1·37	4·42	4·50	7·87	7·58	1·97	—	—	—	3·66
V	$CDCl_3$, 0·25	1·34	1·36	5·25	4·71	—	5·91	—	5·10	2·99	2·80	3·71
VI	$CDCl_3$, 0·25	1·34	1·42	5·05	4·72	—	5·94	—	5·11	3·02	2·83	3·70
VII	$CDCl_3$, 0·25	1·35	1·40	4·46	5·22	7·03	—	2·15	—	2·96	2·83	3·74
	H_2O, ~0·1	1·38	1·42	—	—	8·22	—	2·12	—	2·96	2·80	3·74
VIII	$CDCl_3$, 0·25	1·27	1·40	4·50	5·24	7·02	—	2·15	—	2·91	2·84	3·69
	H_2O, 0·2	1·24	1·31	—	—	8·20	—	2·08	—	2·95	2·80	3·74
IX	$CDCl_3$, 0·25	1·35	1·42	5·16	4·90	—	7·04	1·99	—	3·01	2·85	3·70
	H_2O, 0·2	1·32	1·46	—	—	—	8·17	2·00	—	3·12	2·86	3·73
X	$CDCl_3$, 0·25	1·32	1·44	5·02	4·95	—	7·02	1·99	—	3·04	2·83	3·68
	H_2O, 0·2	1·21	1·31	—	—	—	8·18	1·94	—	3·09	2·79	3·66

compound concentration and the temperature.[10] An upfield shift of the NH-signal occurs with decreasing dipeptide concentration in $CDCl_3$ (Fig. 6). A similar shift occurs on raising the temperature. This provides additional evidence[13] of self-association of the dipeptive molecules in solution.

In the case of the N-acetylated derivatives the chemical shifts of the $N_{(3)}H$ and $N_{(6)}H$ protons in the free state could be assumed to be equal. However, the IR spectra showed that the $N_{(3)}H$ group takes part in intramolecular binding, so that one should expect on extrapolation to infinite dilution the $N_{(3)}H$ proton would be less screened than the $N_{(6)}H$ proton.[14]

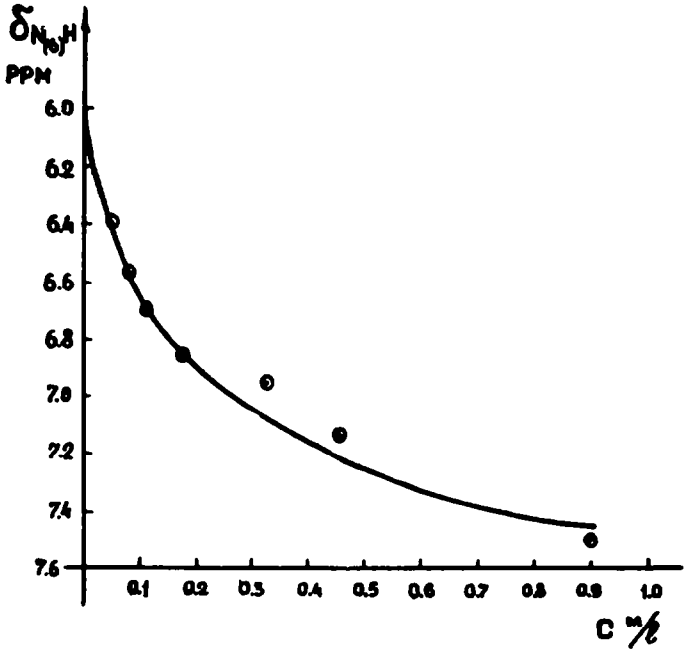


FIG 6 Concentrational dependence of the chemical shift of the $N_{(6)}H$ proton in the NMR-H^1 spectrum of a solution of N-acetyl-D-alanyl-D-alanine methyl ester (III) in $CDCl_3$. The curve has been plotted from data calculated according to the least square method.

Indeed for compound III, as example, the extrapolated $N_{(6)}H$ shift is 6·0 ppm and $N_{(3)}H$ shift is 6·5 ppm. For compounds I and II, for which extrapolation was also carried out, the $N_{(3)}H$ shifts were 6·4 and 6·5 ppm, respectively.

The spectra of the N-Me derivatives (V–X) show the presence of a *cis* methylated amide bond (about 20%). This is particularly clearly seen in the N—CH_3 signals, downfield ($\sim$3 ppm) for the *trans* isomer, and upfield ($\sim$2·8 ppm) for the *cis* isomer (Fig. 3). The assignment was made on the basis of published data.[15] The unmethylated dipeptides contain only *trans*-amide bonds.

The details of the thermodynamic investigation of *cis-trans* isomerization of these compounds will be published elsewhere.

Doublet patterns of the NH signal due to vicinal CH coupling were observed for solutions of the compounds in question in deuterochloroform, dimethylsulfoxide, carbon tetrachloride, acetone and water. In D_2O slow exchange takes place:

$>NH + D_2O \rightleftarrows >ND + HDO$, which hinders observation of the NH signal. The fourth column of Table 4 gives the values for the vicinal spin–spin coupling constants, $^3J_{NHCH}$ of the NH–CH peptide fragment determined at room temperatures from the NH splitting.

Table 5 shows the values of the $J_{N_{(6)}H-CH}$ constants for solutions in CCl_4 and in (1:9) $CDCl_3 + CCl_4$ mixtures for the same concentrations as were used for the IR determination of the folded form fraction. It is noteworthy that in the concentration range of 0·3 moles/l to 0·01 moles/l the value of the coupling constant is practically independent of the concentration.

Table 4. Proton spin–spin coupling constants of the fragment NH—CH and the calculated *cis*-rotamer population x

Compound	Solvent	Coupled proton	$^3J_{NH-CH}$		x	
			observed	corrected	min	max
I	$CDCl_3$	$N^{(3)}H$	7·5	8·1	0·77	1·0
		$N^{(6)}H$	7·6	8·2	0·78	1·0
II	$CDCl_3$	$N_{(3)}H$	7·3	7·9	0·74	0·97
		$N_{(6)}H$	7·6	8·2	0·78	1·0
III	$CDCl_3$	$N_{(3)}H$	7·6	8·2	0·78	1·0
	$(CH_3)_2SO$	$N_{(3)}H$	7·2	7·8	0·73	0·95
	H_2O	$N_{(3)}H$	6·4	6·9	0·60	0·76
	$CDCl_3$	$N_{(6)}H$	7·7	8·3	0·79	1·0
	$(CH_3)_2SO$	$N_{(6)}H$	7·6	8·2	0·78	1·0
	H_2O	$N_{(6)}H$	5·1	5·7	0·44	0·50
IV	$(CH_3)_2SO$	$N_{(3)}H$	7·2	7·8	0·73	0·95
	H_2O	$N_{(3)}H$	7·2	7·8	0·73	0·95
	$(CH_3)_2SO$	$N_{(6)}H$	7·8	8·4	0·81	1·0
	H_2O	$N_{(6)}H$	5·8	6·4	0·54	0·65
V	$CDCl_3$	$N_{(6)}H$	7·7	8·3	0·79	1·0
	CCl_4	$N_{(6)}H$	8·0	8·7	0·85	1·0
VI	$CDCl_3$	$N_{(6)}H$	7·6	8·2	0·78	1·0
	CCl_4	$N_{(6)}H$	7·6	8·2	0·78	1·0
VII	$CDCl_3$	$N_{(3)}H$	6·9	7·5	0·69	0·89
	H_2O	$N_{(3)}H$	6·0	6·6	0·56	0·70
VIII	$CDCl_3$	$N_{(3)}H$	7·3	7·9	0·74	0·97
	H_2O	$N_{(3)}H$	6·5	7·1	0·63	0·81
IX	$CDCl_3$	$N_{(6)}H$	7·1	7·7	0·71	0·93
	CCl_4	$N_{(6)}H$	7·6	8·2	0·78	1·0
	H_2O	$N_{(6)}O$	5·8	6·4	0·54	0·65
X	$CDCl_3$	$N_{(6)}H$	7·0	7·6	0·70	0·91
	CCl_4	$N_{(6)}H$	7·4	8·0	0·75	1·0
	H_2O	$N_{(6)}H$	6·2	6·8	0·59	0·74

TABLE 5. OBSERVED SPIN–SPIN COUPLING CONSTANTS $N_{(6)}H—C_{(5)}H$ PROTONS AND CHEMICAL SHIFTS OF $N_{(6)}H$ PROTON OF THE ALANINE DIPEPTIDES IN DILUTE SOLUTIONS

Compound	Solvent	Conc in moles/l	^3J c/s	δ ppm
I	$CDCl_3 + CCl_4$ (1:9)	0·005	7·6	5·36
		0·012	7·8	5·42
		0·038	7·8	5·60
II	$CDCl_3$	0·030	7·4	5·29
		0·100	7·3	5·40
		0·300	7·4	5·45
III	$CDCl_3 + CCl_4$ (1:9)	0·014	7·6	6·60
	(1:5)	0·031	7·7	6·87
	(1:3)	0·051	7·8	7·04

III DISCUSSION

The IR studies showed that a certain portion of the alanine dipeptide (III, IV) molecules and of their N—Me derivatives are in a hydrogen-bonded folded form. The relative amount of the folded form is about 70% and is independent of the presence of a Me group in the 6 position (Table 2 and Table 4 in Ref. I*b*). Based on these data determination of the preferred conformation of the NH—CH fragment would be made from an analysis of the vicinal NH—CH spin–spin coupling constants.

There are a number of NMR investigations[16] into the conformational states of the side chains of a number of amino acids and dipeptides. However, no attempts had been made to apply this method to the peptide system's main chain which is of major importance in determining the spatial structure of polypeptides and proteins.

(a) *Rotational state models of the* CONH—CH *bond*

The limited data on the stereochemical dependence of the $^3J_{CONH—CH}$ constant in amide systems[15b, 17, 18] and the very incomplete knowledge of the stable conformations of the NH—CH fragment[2a–d, 3a–k, 19] do not allow of a straightforward approach to the spatial structure of the dipeptide chain.* Nonetheless a certain indication of the conformational states of the dipeptides in solution can be obtained on the basis of plausible assumptions and models for the rotational states of the NH—CH bond.

Since both nitrogen and carbon electronic shells contain only *s* and *p* orbitals, in a theoretical treatment of the transmission of spin–spin coupling these atoms can be considered to be equivalent.[20, 21] Therefose in elucidating the stereochemical dependence of the spin coupling constant for the OC—NH—CH—R_1R_2 protons, use can be made of the generalities established for the C=CH—CH[21, 22a–d] fragment.

* It is difficult to discuss the available literature data on the $^3J_{NHCH}$ coupling constants in connection with the geometry of the —NH—CH< bond because the compounds investigated are very flexible and their conformation is uncertain.

These are namely:

(1) the dependence of the ^3J vicinal proton coupling constant upon the dihedral angle θ between H—N—C— and N—C—H— planes which is given by the expression:

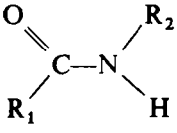
$$^3J = A\cos^2\theta - B\cos\theta + C\sin^2\theta, \tag{1}$$

where A, B and C are positive coefficients with $A > C > 0$ and $A > 2B > 0$. The first two members of the equation represent the coupling transmitted through the σ bonds.[20] The last term stands for the contribution due to the π-electron orbital of the one of the atoms in the fragment.[22b] Hybridization of the valence orbitals, bond length and valence angles do not change the form of Eq. (1), affecting only the values of the coefficients A, B and C,[20]

(2) the effect of the electronegativity of the substituents on the atoms of the fragment which is approximated by the relation[21, 22a, c, d]

$$J_{corr} = J_{obs}(1 - \alpha\sum_i \Delta E_i)^{-1} \tag{2}$$

where J_{corr} is the corrected value for the constant, J_{obs} the observed value, ΔE_i the electronegativity difference between the R_1 substituent and hydrogen, and α — a proportionality coefficient. The value of α was taken to be 0·1 from a set of values for the $\rangle$CH—CH$\langle$ [22c, d] fragment. The values of J_{corr}, corrected for the electronegativity of the substituent are given in column 5 of Table 4.

In constructing a conformational model of a dipeptide we assumed that the planar *trans*-amide bond  is the stable form in solution. The conformation of this bond has been fully investigated by a number of physicochemical methods.[2a, c, d, 15, 19]

We shall discuss the rotation about the —CONH—CH$\langle$ bond in terms of two models.

(*a*) *Model with discrete rotational states.* Usually in treating the internal rotation about a bond between trigonal and tetrahedral C atoms it is assumed that one of the bonds of the sp^3 carbon is in eclipsed position with respect to the double bond of the sp^2 atom,[2d, 23] while the two other bonds of the sp^3 carbon form projection angles of 120° with the double bond. Since the NH—CH bond in question is also formed by a trigonal atom (N) and a tetrahedral atom (C), as a first approximation one may consider that the dihedral angle θ for the N—C rotational isomers can assume only the discrete values[19, 3c] of 0° (*cis* arrangement of the protons), 60 and 300° (gauche), 120 and 240° (gauche) and 180° (*trans*). Then from Eq. (1) we obtain:

$$\begin{aligned} ^3J_{cis} &= A - B \\ ^3J_{trans} &= A + B \\ ^3J_{gauche\text{-}300°} &= {}^3J_{gauche\text{-}60°} = \tfrac{1}{4}(A - 2B + 3C) \\ ^3J_{gauche\text{-}240°} &= {}^3J_{gauche\text{-}120°} = \tfrac{1}{4}(A + 2B + 3C) \end{aligned} \tag{3}$$

Small deviations ($\pm 10°$) of θ from the chosen values have no essential bearing on the results of the calculations to follow.

For the NMR based treatment of rotational isomerism about single bonds, one must have knowledge of the rotational potential curve. Such information with respect to the NH—CHR_1R_2 peptide fragment is lacking even in the simplest case $R_1 = R_2 = H$.[2a,d,19] Different variants of internal rotation model about this bond must therefore be analysed.

For the NH—CH_3 fragment of N-methyl formamide in general three most profitable models are possible (Fig. 7): (*a*) *cis* rotation: (*b*) *trans* rotation and (*c*) *cis-trans*

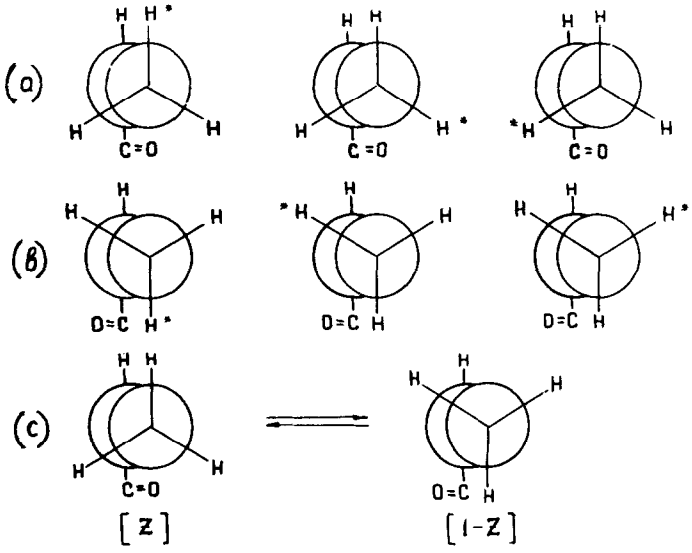


FIG. 7. Model representation of the CONH—CH_3 rotational isomerism in N-methyl-acetamide: (a) *cis* rotation, (b) *trans* rotation, (c) *cis–trans* rotation.

rotation. The mean values of the coupling constants $\langle J \rangle$ are given by the following relations:

$$\begin{aligned} \langle J \rangle_a &= (\tfrac{1}{3})(J_{cis} + 2J_{gauche\text{-}120^\circ}) \\ \langle J \rangle_b &= (\tfrac{1}{3}))(J_{trans} + 2J_{gauche\text{-}60^\circ}) \\ \langle J \rangle_c &= Z\langle J \rangle_a + (1 - Z)\langle J \rangle_b \end{aligned} \tag{4}$$

where Z is the *cis* rotamer population of model *c* (Fig. 7*c*).

On substituting Eq. 3 we find that all three models give the same values for the constants:

$$\langle J \rangle_a = \langle J \rangle_b = \langle J \rangle_c = (\tfrac{1}{2})(A + C) \tag{5}$$

The most reliable experimental value for $^3J_{NHCH_3}$ is 4·9 ± 0·1 c/s[18] so that

$$A + C = 9{\cdot}8 \text{ c/s} \tag{6}$$

For the peptide fragment CONH—CHR_1R_2 two internal rotation models (Fig. 8) are considered: (*a*) *cis* rotation and (*b*) *trans* rotation. The spin–spin coupling constants for these models are given by the following equations:

$$J_A = xJ_{cis} + (1 - x)J_{gauche\text{-}120^\circ} \tag{7}$$

$$J_B = yJ_{gauche\text{-}60^\circ} + (1 - y)J_{trans} \tag{8}$$

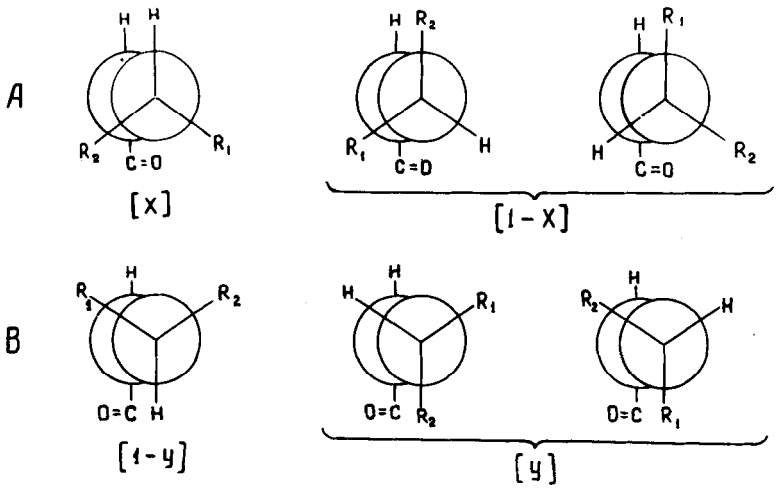


FIG. 8 Model representation of the CONH—CHR_1R_2 rotational isomerism in peptides: (a) *cis* rotation, (b) *trans* rotation.

where x is the population of the rotamer with the protons *cis* and y the rotamer with the protons *gauche*-60°.

Substituting Eq. (3) and taking into account the Eq. (5) we obtain:

$$J_A = (\tfrac{1}{2})[\langle J\rangle(3x + 1) + (B + C)(1 - 3x)] \tag{9}$$

$$J_B = (\tfrac{1}{2})[(A + B)(2 - 3y) + 3y\langle J\rangle] \tag{10}$$

In order to determine the regions of permissible values for the parameters $B + C$ and $A + B$ we plot the functional curves $(B + C) = f(x)$ and $(A + B) = f(y)$. The corresponding diagrams are given on Fig. 9 for $\langle J\rangle = 4{\cdot}9$ c/s and $J = 8$ c/s. The value of J is within the bounds of the observed values for the coupling constants of the compounds investigated with account of the electronegativity of the substituents (see column 5 of Table 4 and Table 5). For correction of the substituent effect E_H was taken[24] to be 2·1 and $E_C = 2{\cdot}5$.

From analysis of the diagrams on Fig. 9 with account of the Relation (6) and the initial assumptions of $A > 2B > 0$ and $A > C > 0$, it follows that the inequality $0 < (B + C) < 1{\cdot}8$ c/s should obtain for model A and $8{\cdot}0 < (A + B) < 14{\cdot}7$ c/s for model *B*. If, now, the Relations (9) and (10) be plotted with the limiting values for $(B + C)$ and $(A + B)$ it will be possible to estimate the probable population of the rotamers x and y for peptide systems with known values of J. The corresponding diagrams are given in Fig. 10.

The IR investigations discussed in the present paper showed that in CCl_4 solution from 60 to 80% of the alanine dipeptide molecules and their $N_{(6)}$-Me derivatives are in the hydrogen-bonded folded form.

Examination of molecular models shows that the formation of such an internal hydrogen-bonded 7-membered ring is possible only under the condition that the N_6H—C_5H rotamer is in either the *cis* ($A[x]$), (Fig. 8) or one of the *gauche*-120°

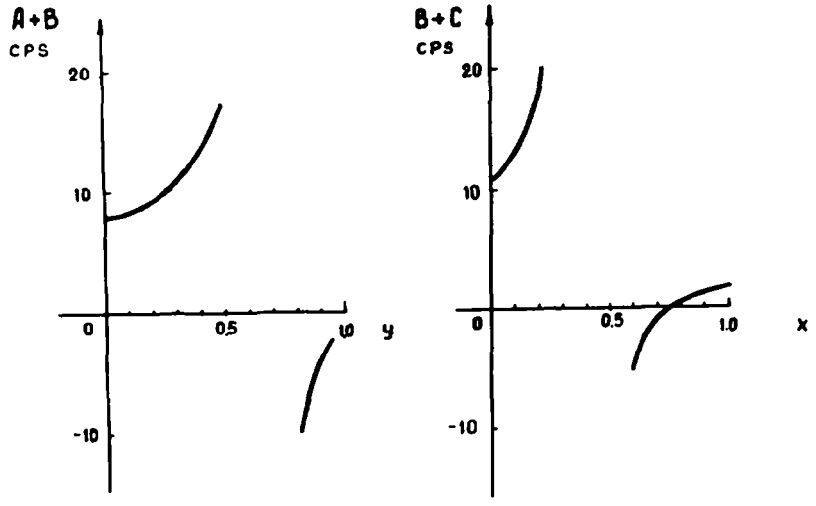


FIG. 9 Dependence of (A + B) and (B + C) upon the rotamer population y and x (see Eqs 9 and 10, respectively) for the discrete rotation state model.

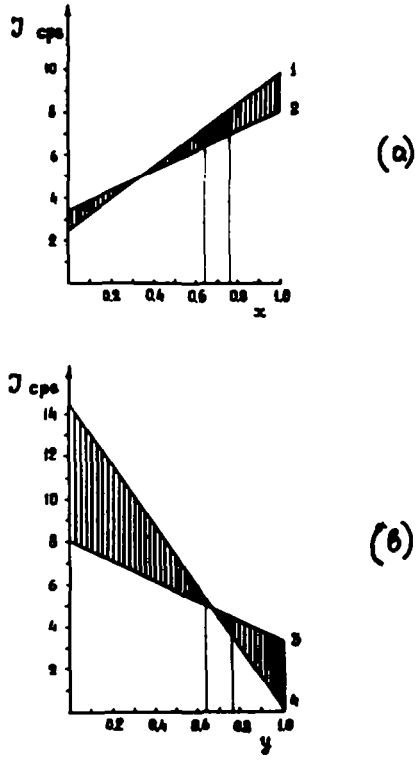


FIG 10 Dependence of the $^3J_{NH-CH}$ constants on the rotamer population x and y for the discrete rotation state model. The hatched portions represent the areas of permissible values of J

($A[1-x]$) or one of the *gauche*-60° ($B[y]$) conformations. In order to establish which type of the N_6H—C_5H rotamer is the preferred one we turn to the vicinal proton spin–spin coupling constants of this fragment. It was found for the DD isomer of the alanine dipeptide (III) in $CDCl_3$ solution either a 75–100% *cis* rotamer content (Fig. 10a) or a 0–45% *gauche* rotamer content (Fig. 10b). Since the folded form fraction of this dipeptide $n = 0{\cdot}74$ (Table 2 and Table 4 in Ref. 1*b*) it is evident that only the *cis* rotamer model conforms to the whole of the results obtained. From this it follows that the stereochemical dependence of the spin coupling constants of the vicinal CONH—CH protons is determined by the relation:

$$^3J_{NHCH}\,(c/s) = (8{\cdot}9 \pm 0{\cdot}9)\cos^2\theta - (0{\cdot}9 \pm 0{\cdot}9)\cos\theta + (0{\cdot}9 \pm 0{\cdot}9)\sin^2\theta \quad (11)$$

The low value of the coefficient C for the CONH—CH< fragment of the peptide as compared to that for the C=CH—CH< fragment ($C = 2{\cdot}6$[22b]) is in agreement with the fact that the unshared electron pair of the nitrogen is only partially delocalized, and therefore the π-contribution to the coupling constant should not be very great.

In Fig. 11, for comparative purposes plots are given of the stereochemical dependence of the vicinal proton coupling constants for the fragments CONH—CH< and C=CH—CH<.[22b] On the basis of Eq. (9) the *cis* rotamer population x for solutions of the dipeptide have been calculated and included in Table 4.

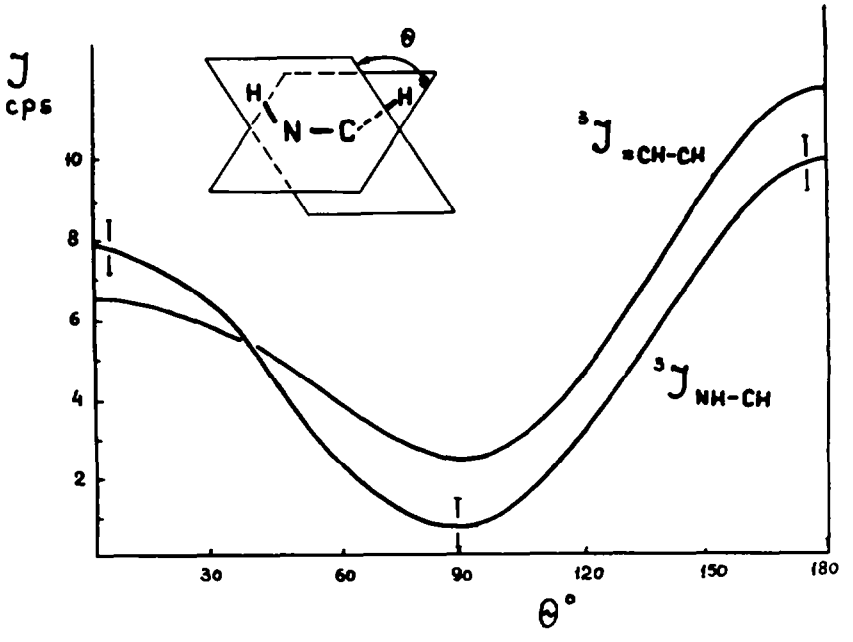


FIG. 11 Stereochemical dependence of the vicinal proton coupling constants of the CONH—CH< and C=CH—CH< fragments. θ—the dihedral angle between the H—N—C and C—C—H planes.

(*b*) *The free rotating model.** An alternative model for the internal rotation about the CONH—CH< bond could be one of free rotation (since the sp^2/sp^3 potential barriers are markedly lower than the sp^3/sp^3 barriers), the energy minima of which are determined by steric factors.

On free rotation of the Me group of the CONH—CH_3 fragment the mean value of the coupling constant is

$$\langle J \rangle = \int_0^{2\pi} J(\theta)\, d\theta \Big/ \int_0^{2\pi} d\theta = (A + C)/2, \tag{12}$$

in agreement with the results obtained for the discrete states model. We shall assume that the observed coupling constant J of the $N_{(6)}H$—$C_{(5)}H$ fragment of alanine dipeptides whose molecules take part in the folded (hydrogen-bonded) extended form equilibrium can be expressed by the relation

$$J = nJ_{fold} + (1 - n)J_{ex},$$

where n is the fraction of folded form as determined by IR spectroscopy, J_{fold} is the coupling constant for the folded form and J_{ex} is the constant for the extended form. Presenting J_{fold} in Eq. (1) as function of the dihedral angle θ and taking into consideration the Relation (12) we obtain:

$$\cos\theta = \frac{B \pm \sqrt{\{B^2 + 8[A^2 + Ar - q]\}}}{4(A - \langle J \rangle)},$$

where

$$r = \frac{J - (1 - n)J_{ex} - 3\langle J \rangle n}{n}$$

and

$$q = \langle J \rangle \left(\frac{J - (1 - n)J_{ex}}{n} - 2\langle J \rangle \right)$$

The assumption made above with respect to the values of the coefficients in Eq. (1) give the following regions of values:

$$4{\cdot}9 \leqslant A \leqslant 9{\cdot}8; \qquad 0 \leqslant B \leqslant A/2. \tag{13}$$

For the $N_{(6)}H$—$C_{(5)}H$ fragment of dipeptide (III) the value of the constant is $J_{corr} = 8{\cdot}3$ c/s. For J_{ex} of the extended form we assume the value $J_{corr} = 7{\cdot}7$ c/s obtained with the $N_{(3)}$-methylated derivative (IX) of this dipeptide, since compound (IX) cannot form intramolecular hydrogen-bond and should therefore be in the extended form. Under comparable conditions the proportion of folded form for compound III was found by IR spectroscopy to be $n = 0{\cdot}75$. For differing fixed values of A a range of B values limited by the inequality (13) results in the range of θ values. The hatched areas (Fig. 12) show the regions of permissible values of θ for the $N_{(6)}H$—$C_{(5)}$ fragment of molecules with the folded forms: $|\theta| \leqslant 22°$ and $180° \leqslant |\theta| \leqslant 135°$.

* The authors are very grateful to the anonymous referee for pointing out the necessity of considering the free rotation model and also for his valuable advice and comments.

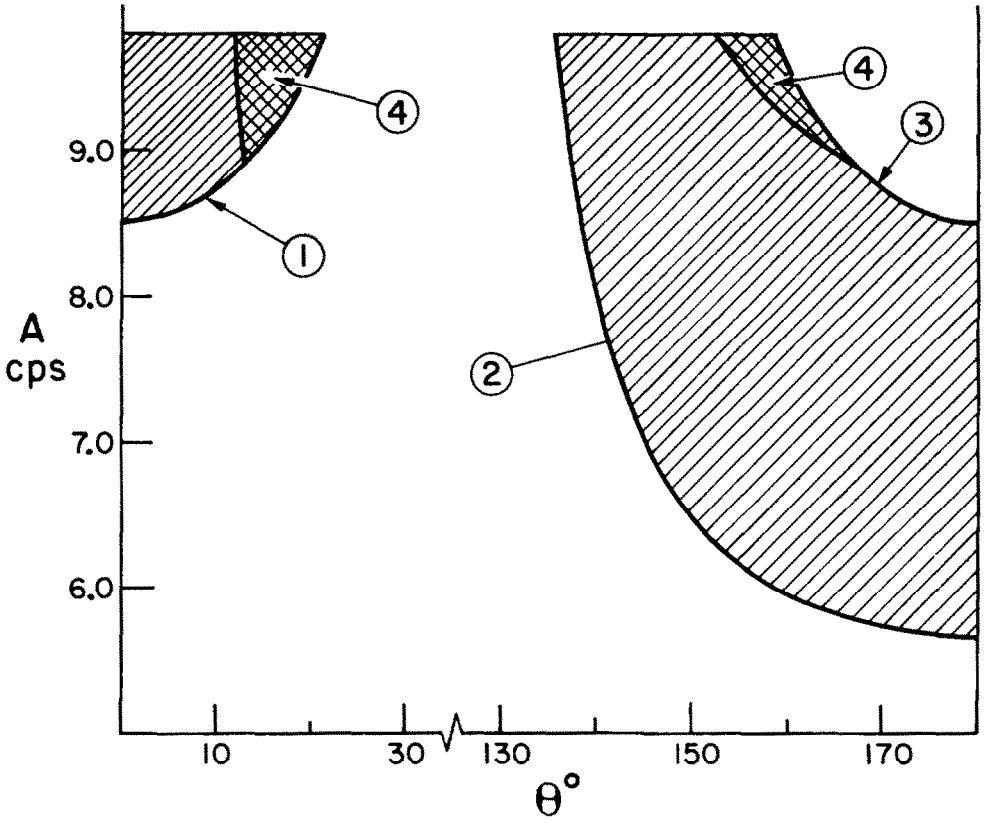


FIG. 12 Region of allowed θ values for the free rotation model of the —CONH—CH⟨ fragment (see the text).

In an NMR-H^1 study of 5,6-dihydrouracils French authors[17e–i] have found that the value of the $^3J_{NHCH}$ constant is 3·8–4·9 c/s for the equatorial CH proton and 1–1·4 c/s for the axial proton. For 6-phenyldihydrouracil ^3J = 8·2 c/s.[17i] Although the conformation of the latter could not be established with certainty, it could be assumed that such a value indicates an equatorial proton.* These data make it possible to restrict the areas of possible values for the θ angles, since obviously in order to conform with the above established condition that $J_{eq} > J_{ax}$ the requirement must be fulfilled that $B \leqslant A - J_{eq}$. Introducing the substituent electronegativity correction upon Eq. (2) we obtain the maximum value for J_{corr} = 8·9 c/s and the regions of permissible values are therefore $12° \leqslant |\theta| \leqslant 22°$ and $167° \geqslant |\theta| \geqslant 152°$. These regions (4) have been cross-hatched in Fig. 12. Under such conditions the values of the coefficients A, B and C in Eq. (1) coincide with those derived on the basis of the discrete state internal rotation model (Eq. 11).

* In spite of such a high value of $^3J_{NHCH}$ being only once mentioned by French authors, this value is very significant in the relation to the very possible shift of the conformational equilibrium to the most stable form of this molecule. Moreover we have obtained recently values of $^3J_{NHCH}$ equal to about 7·5–8·0 c/s for some cyclic peptides. This data will be published elsewhere in the near future.

An examination of molecular models shows that intramolecular hydrogen-bonding which provides for the folded form stability occurs without difficulty at values of $|\theta| < 20°$. However, with $|\theta| > 150°$ considerable strain should arise, weakening the hydrogen-bond and consequently lowering the preference for the folded form. From this it follows that both models for the internal rotation of the —CONH—CH< peptide fragment discussed in the light of NMR and IR data lead to the same conclusion: preference of the *cis* orientation of the hydrogen atom in the above fragment.

(b) *Conformation of the folded form of dipeptides*

The preference for the *cis* $N_{(6)}H—C_{(5)}H$ rotamer in the 7-membered ring determines the type of $C_{(5)}—C_{(4)}$ rotamer bond in this ring. From the fact of the existence of an intramolecular hydrogen-bond it follows that the CO group should be eclipsed by the $C_{(5)}H$ hydrogen. The preferred conformation of the 7-membered ring with the aforementioned rotational isomers of the $N_{(6)}—C_{(5)}$ and $C_{(5)}—C_{(4)}$ fragments is depicted on Fig. 13b. On the conformation charts[3k] this conformation corresponds to the region $\phi_{N—C\alpha} \sim 240°$ and $\psi_{C^{\alpha}—C} \sim 120°$. Such a 7-membered ring corresponds to the conformation of the polymer chain fragment proposed by Huggins.[25]

One should mention that this region has no energy minimum in the charts.[3k] But it should be borne in mind that the charts were plotted with account only of steric and electrostatic interactions in the peptide residue. The possibility of the formation of intramolecular hydrogen-bonds between the atoms O_{i-1} and H_{i+1}[3k] as in the

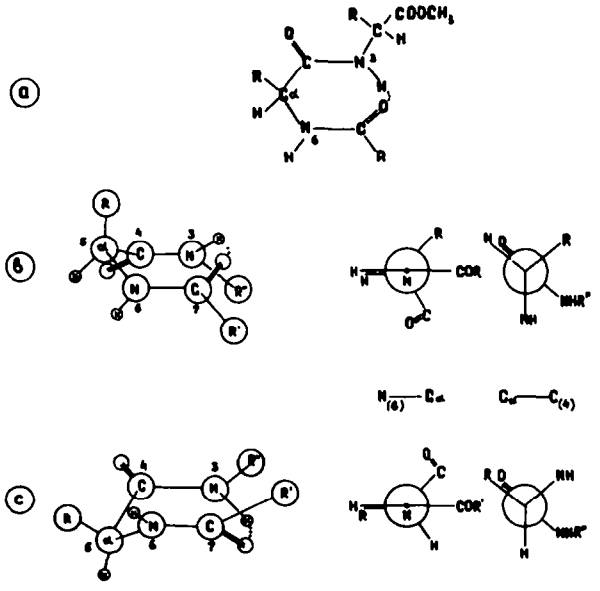


FIG. 13 The preferred conformation of dipeptide fragment. (a) The intramolecular hydrogen-bond with 7-membered ring. (b) The preferred conformation of the α-alanine dipeptide fragment and the preferred rotational N—C^{α} and C^{α}—C isomers. (c) Conformation of the fragment as proposed by Mizushima.[2d, 6]

case of the 7-membered ring discussed here was left our of consideration. Obviously, therefore, the nearing of the O_{i-1} and H_{i+1} atoms close enough for hydrogen-bond formation would correspond in the calculations to increased energy of the molecules due, for instance, to steric interaction between these atoms. One may suppose that calculation with account of the possibility of intramolecular hydrogen-bonding between O_{i-1} and H_{i+1} would show an energy minimum in the region around $\phi \sim 240°$ and $\psi \sim 120°$, the more so that steric and electrostatic factors permit the existence of such rotamers about separate N—C^{α} and C′—C^{α} bonds.

An alternative conformation of the folded form is that proposed by Mizushima on the basis of general considerations.[2d, 19, 26]

This model (Fig. 13c) assumes a *gauche* ($\theta \sim 120°$) position of the H atoms in the $CON_{(6)}H—C_{(5)}H$ fragment and corresponds to the region in the conformation charts of about $\phi \sim 120°$ and $\psi \sim 240°$. The present NMR and IR study shows, however, that such conformation of the peptide fragment would be very improbable owing to considerable deviation (more than 32°) of the angle θ from the value most advantageous for hydrogen-bonded closure of the 7-membered ring.

Support for the *cis* isomer model is to be found in retention of the fraction of the folded form in the $N_{(6)}$-methylated dipeptides. Here the *gauche*-120° rotamer would have eclipsed methyls of the $N_{(6)}—CH_3$ and $C_{(5)}—CH_3$ groups, a highly disadvantageous arrangement because of steric hindrance.

(c) *Conformation of the extended form of the dipeptides*

The stereochemical dependence of $3J_{NHCH}$ constant upon the dihedral angle θ of the CONH—CH< fragment derived on the basis of the conformation established for the folded form of the dipeptides permits to discuss the possible preferred conformations of the extended form in solutions.

It is noteworthy that values of the proton coupling constants of the $N_{(6)}H—C_{(5)}H$ fragment (an element of the 7-membered ring) and of the $N_{(3)}H—C_{(2)}H$ fragment which can be regarded as the side chain of this ring) are about the same in $CDCl_3$ and CCl_4 solutions (Tables 4 and 5). It is quite plausible to assume that this similarity is indicative of preference for a *cis* arrangement of the CONH—CH bond not only in the hydrogen-bond stabilized ring, but also in the side chain. Hence, quite possible in inert solvents the stability of this rotamer is determined by the potential curve of internal rotation of this bond. In that case the preferred conformation of the extended form of alanine dipeptides corresponds to ϕ 240°, the permissible value of the C^{α}—C bond angle from the conformation charts is the region ψ 240° corresponding to a left-handed helix. It is, however, not excluded that in inert solvents the preferred rotamer of the CONH—CH bond in the extended form of the dipeptides is that with the dihedral angle $166° \leqslant |\theta| \leqslant 152°$ (Fig. 12). Judging from the conformation charts,[3] in that case the stable conformation would correspond either to the region ϕ 90° and ψ 250° or $70° \leqslant \phi \leqslant 90°$ and ψ 120°.

The value of the NH—CH proton coupling constant (Table 4) decreases in polar solvents (dimethylsulphoxide and water). From all the above-said this should mean that certain destabilization of the *cis* NH—CH rotamer takes place. The reasons for such destabilization could be: first, rupture of the intramolecular $C_{(7)}O \ldots H—N_{(3)}$ bond by these proton accepting and donoring solvents and secondly, change in the preferred conformation of the entire dipeptide chain. Since change occurs in both

$^3J_{N_{(3)}H—C_{(2)}}H$ and $^3J_{N_{(6)}H—C_{(5)}H}$ constants, it is probable that both factors affect the relative population of the rotamers with respect to the NH—CH bond.

The decrease in value of the ^3J constant indicates that considerable weight is acquired by the conformer with the angle θ close to 60 or 120°. On the basis of the conformation charts more probable is the increase in weight in polar media of the conformation ϕ 120, ψ 120° corresponding to the right-handed helix.

The largest effect is observed for aqueous solutions, with the fall in J greater for the folded form of the $N_{(6)}H—C_{(5)}H$ fragment (ΔJ = 2·6 c/s) than for the extended form of the $N_{(6)}H—C_{(5)}H$ and the $N_{(3)}H—C_{(2)}H$ fragments (ΔJ = 1·3–0·8 c/s) with reference to solutions in $CDCl_3$ (Table 4). Hence the $N_{(6)}H—C_{(5)}H$ fragment forming part of the intramolecular hydrogen-bonded 7-membered ring, which exists in inert solvents, apparently undergoes considerably larger conformational change in water than does the $N_{(3)}H—C_{(2)}H$ side chain.

On the basis of the Kirkwood model for the electrostatic energy of solvation of a dipole μ in polar solvents[27] it follows that the gain in free energy ΔG is

$$\Delta G = -\frac{\varepsilon - 1}{2\varepsilon + 1} \cdot \frac{\mu}{r^3}$$

where ε is the dielectric constant of the medium, and r the radius of the molecular cavity of the dipole. Therefore the more polar form of the molecule is energetically more advantageous in a medium with a higher dielectric constant. For dipeptides this form would be the extended chain. Chain extension is effected by rotation about N—C^α and C^α—C'(O) bonds. The *cis* NH—CH isomer population calculated from Eq. (7) (Table 4) for $CDCl_3$ solutions (ε = 4·81) is about 80–90%; for $(CH_3)_2SO$ (ε = 45·0) it is about 70–80% and for aqueous solutions (ε = 82) it falls to 40–60%. Besides the increase in dielectric constant the more significant conformational changes in aqueous solutions is apparently caused by specific molecular interaction such as, for instance, formation of an intermolecular hydrogen-bond network. Since, however, preference for the *cis* isomer is still retained, one may expect that the main contribution to straightening of the dipeptide is rotation about the C—C(O) bond.

The authors are deeply grateful to the anonymous referee for his careful discussion of this work and his many important remarks and suggestions.

CONCLUSIONS

1. In carbon tetrachloride solution about 70% of alanine dipeptide molecules with the exception of $N_{(3)}$—Me derivatives are in the form of an intramolecular hydrogen-bonded 7-membered ring.

2. A preferred conformation with *cis* orientation of the H atoms CONH—CHRR' has been proposed for the 7-membered hydrogen-bonded ring (Fig. 13b).

3. A stereochemical dependence has been found for the vicinal proton spin–spin coupling constant of the CONH—CH fragment.

4. NH—CH fragments not forming part of the 7-membered hydrogen-bonded ring and also the extended forms of the $N_{(3)}$-methylated dipeptides can be in the conformation with $\theta \sim 160°$ (ϕ 80°) as well as in the cisoid form $\theta \sim 0°$ (ϕ 240°).

5. There is a considerable increase in population of the $\theta \sim 60°$ (ϕ 120°) rotational state for the CONH—CH$\lessdot$ fragment in polar media, in particular, in aqueous which probably corresponds to a right-handed helix.

EXPERIMENTAL

The NMR spectra were obtained on JEOLCO JNM-C60 and JNM-4H-100 spectrometers operating at 60 and 100 Mc/s, respectively. TMS was used as internal reference. The spin–spin coupling constants were measured with a sweep width of 1 ppm; the results are the average of eight determinations, the experimental error being as a rule $\pm 0{\cdot}1$ c/s (standard deviation). The double resonance technique used is described in detail.[28] The NMR-H^1 spectrum for the highly dilute dipeptide solutions ($5 \cdot 10^{-3}$–$5 \cdot 10^{-4}$ moles) were taken under slow sweep conditions (sweep rate 0·03–0·01 c/s/sec) utilizing a high response time (10–30 sec) for the phase sensitive detector. For such low concentrations the error in determination of J is from $\pm 0{\cdot}2$ to $\pm 0{\cdot}3$ c/s.

Because the components of the NH doublet were quite wide (halfwidth from 3 to 6 c/s) a correction to the inter-doublet spacing was made under the assumption of Lorentzian line shapes. The magnitude of the correction depends upon the ratio of the splitting to the halfwidth and in our case varied from 0·2 to 0·4 c/s. In Tables 4 and 5 there are presented values for J_{obs} with these corrections

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