THE PROBABILITY OF THE OCCURRENCE OF *CIS*-PEPTIDE UNITS IN PROTEINS AND POLYPEPTIDES*

ALOK K. MITRA

(*Molecular Biophysics Unit, Indian Institute of Science, Bangalore* 560012)

Received on July 22, 1976 and in revised form on September 7, 1976

ABSTRACT

The problem of the very rare occurrence of cis *peptide units in proteins and polypeptides has been investigated and the probability of occurrence of such peptide units has been evaluated on the basis of conformational analysis. The higher intrinsic energy of the* cis *over the* trans *form is partially responsible for the rare occurrence of the former. We find that the conformational restrictions produced when a* cis *peptide unit occurs within an all-*trans *polypeptide chain provides an additional criterion. Conformational energy calculations, on a segment of two peptide units (called dipeptide for short) indicated that, relative to the* trans-trans *structure, the* trans-cis *or* cis-trans *structures can occur only to an extent of* 0·3 *per cent. In the case of a tripeptide unit, calculations on the configurations* trans-trans-trans *and* trans-cis-trans *yielded for the non-prolyl and prolyl* cis *peptide units a probability of occurrence of about* 0·1 *per cent and* 30 *per cent or higher, respectively. This explains the almost total absence of nonprolyl* cis *peptide units in polypeptide chains. On the other hand, the results indicate that the population of* cis-*Pro for the sequence* X-*Pro in globular proteins is likely to be appreciable and that these should be looked for. So also, in the non-prolyl case,* cis *peptide units are not forbidden, but may occur in rare cases, which should also be looked for.*

Keywords: *cis*-peptide unit; polypeptides, prolyl peptide unit; partition function; frequency of *cis* residues.

INTRODUCTION

The building block of polypeptides and proteins is the peptide unit. This, because of the partial double bond character of the CO—NH bond occurring in it, can take up two isomeric modifications—namely, the *cis* and the *trans* peptide units (Fig. 1). However, in recent years, experimental techniques like X-ray crystallography, probing into protein and

* Contribution No. 84 from the Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India.

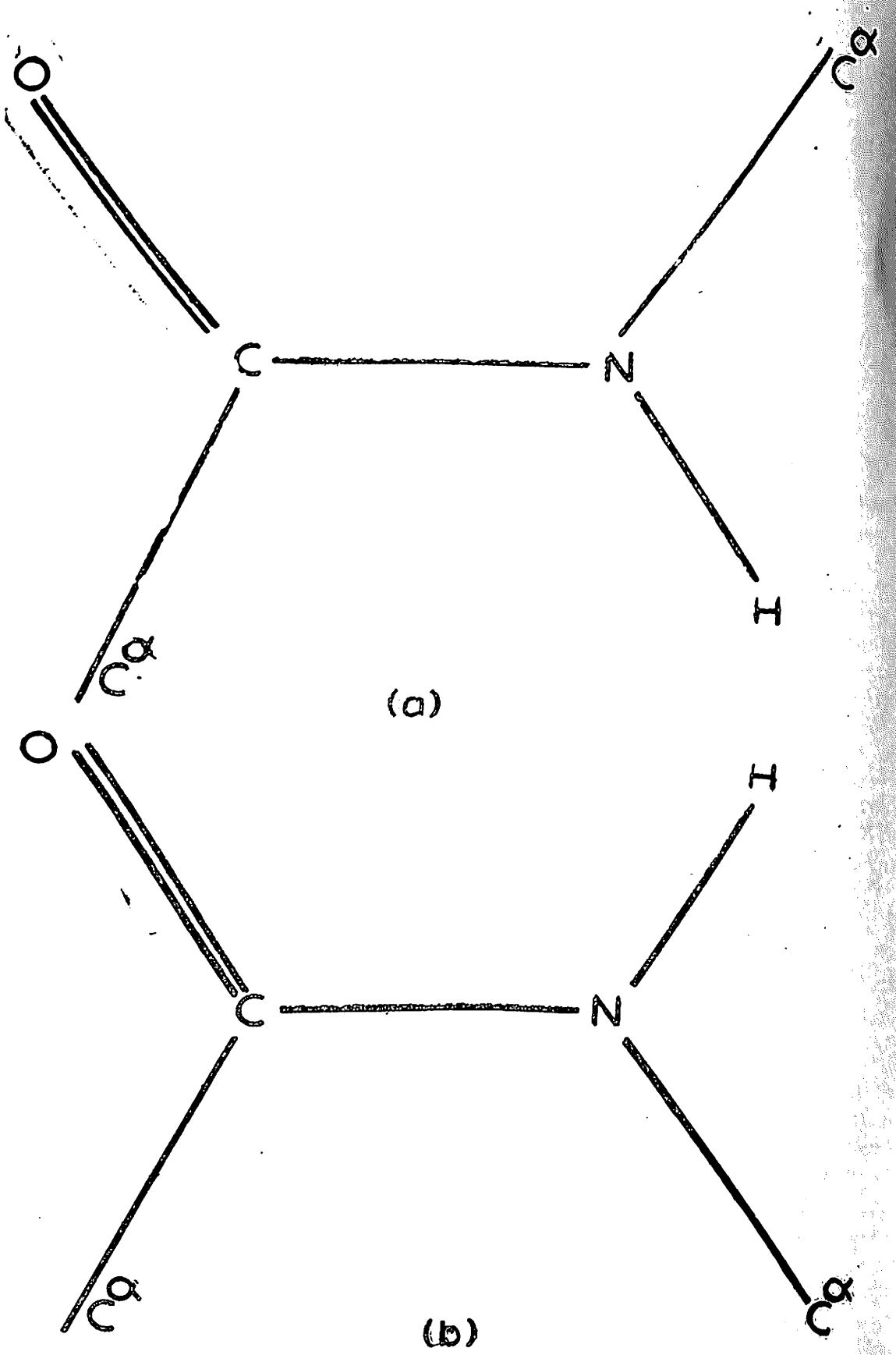

FIG. 1. (*a*) *Trans* and (*b*) *cis* isomers of a peptide unit.

polypeptide structures, have revealed that, of these two, the *trans* form predominates overwhelmingly. Only in special cases, where the peptide unit *X*-*Y* has *Y* as the residue of an *N*-substituted amino acid (such as proline) is the *cis* conformation ever observed. In this paper, we report our investigations of this phenomenon from a theoretical standpoint, employing conformational analysis.

A factor that is definitely responsible for the difference in *cis* and *trans*-population is the intrinsic energy difference between the two. This has been arrived at from theoretical as well as experimental studies on analogues of a peptide unit like N-methyl formamide and *N*-methyl acetamide. Some typical results for the energy difference $\triangle E = E_{cis}\text{-}E_{trans}$ are indicated in Table I. It can be seen that the energy of the *cis* form is higher than that of the *trans* form, although the actual value of the energy difference differs according to the method adopted.

Because of its higher intrinsic energy, the *cis* peptide unit has a lower probability of occurrence (*see* Table I), but this factor alone cannot account for the overwhelming preponderance of the *trans* form. This paper deals with this problem from the point of view of conformational analysis and suggests an explanation for the rarity of *cis* peptide unit in proteins and polypeptides. This has been arrived at from the observation that the presence of a *cis* peptide unit in a polypeptide chain introduces, locally, considerable stereochemical restrictions. Thus, in a dipeptide, the simplest model for a polypeptide, our calculations indicated that the *trans-trans* structure is thermodynamically more favourable than either *trans-cis* or *cis-trans* structure. Next in a tripeptide, a better model for a polypeptide, we found that the probability of the peptide unit in the middle being *cis* is much less than of its being *trans* due to stereochemical constraints. This was arrived at by analysing the tripeptide fragments *trans-trans-trans* and *trans-cis-trans*. On including the intrinsic energy difference between the *cis* and *trans* forms of the peptide unit into our calculations for the tripeptide systems, it may be stated here that the probability of *cis* peptide unit occurrence was found to lie in the range of 0·02 per cent to 0·2 per cent. This result, without significant alteration, yields the probability of finding a *cis* peptide unit in an all-*trans* polypeptide chain. For the specific case of proline, however, similar calculations showed that in the tripeptide fragment a *cis* linkage for the sequence X-Pro is about as probable as *trans*, very much unlike in the non-prolyl case.

TABLE I

Typical values for cis-trans *energy difference of peptide analogues from theoretical and experimental studies and the relative probability of the two isomers computed therefrom*

Workers	Peptide analogue	Method	Energy difference E (kcal/mole)	Relative probability of *cis* form $= \exp(-E/RT)$
		Theoretical		
Murthy *et al.* [1]	N-methyl acetamide	CNDO/2	1·8	0·05
Pericaudet and Pullam [2]	N-methyl formamide	*ab initio* quantum chemical	1·9	0·04
Andrews [3]	N-methyl formamide	PCILO	0·7	0·33
Shipman and Christofferson* [4]	N-methyl acetamide	*ab initio* quantum chemical	2·5	0·015
		Experimental		
Neumann *et al.* [5]	N-methyl formamide	NMR	1·4	0·10
Drakenberg *et al.* [6]	N-methyl formamide	NMR	1·6	0·7
	N-methyl acetamide	NMR	2·8	0·01
Khetrapal [7]	N-methyl formamide	PMR liquid crystalline nematic phase	1·5	0·08

* Shipman and Christofferson made calculations for different orientation of the CH_3 groups and obtained different values for energy difference. The lowest value reported is given here.

METHOD

Our approach to the problem of understanding the reason for the low occurrence of *cis*-peptide units was to study the stereochemical role of such a peptide unit in a polypeptide chain and to see how this differs from that of the *trans* peptide unit.

1. *Dipeptide system*

In order to carry out this objective, we first took a dipeptide as a model system, with one of the peptide units in *cis* conformation, and compared its thermodynamic properties with the case in which both the peptide units are in *trans* conformation. We studied the segment consisting of the atoms C_1^{α}—C_1O_1—N_2H_2—C_2^{α} (H_2^{α}, R_2)—C_2O_2—N_3H_3—C_3^{α} with $R_2 \equiv CH_3$, corresponding to the amino acid alanine. We call this an alanine dipeptide for convenience. For each of the three dipeptide configurations, namely *trans-trans*, *cis-trans* and *trans-cis*, the conformational energy was obtained as a function of the relevant dihedral angles (ϕ, ψ)*. Also, important thermodynamic quantities like partition function (Z), average energy ($\langle E \rangle$) and free energy (F) were computed for comparison.

2. *Tripeptide system*

The stereochemical features accompanying a *cis* peptide unit, when it is present in an all-*trans* polypeptide chain, are evident in its interaction with the first neighbour *trans* peptide units. We therefore considered a system composed of three peptide units, called for convenience "a tripeptide unit", as a better model than the dipeptide system, in further calculation of analysing the influence of the *cis* peptide unit in a protein or polypeptide chain. Configurations of the tripeptide unit were generated by assigning either *trans* or *cis* geometry for the middle peptide unit and *trans* geometry for the two terminal peptide units. We will call these two tripeptide configurations, namely, *trans-trans-trans* and *trans-cis-trans*, as T- and C-configurations, respectively, for the sake of brevity. This study would indicate the relative ease with which a single *cis* peptide unit can occur in a polypeptide chain consisting of *trans* units.

Conformation energy calculations were performed for the tripeptide unit composed of the atoms C_1^{α}—C_1O_1—N_2H_2—C_2^{α} ($H^{\alpha}{}_2$,R_2)—C_2O_2 N_3H_3—C_3^{α}(H_3^{α},R_3)—C_3O_3—N_4H_4—$C_4{}^{\alpha}$ for both T- and C- configurations. In the first set, Ala-Ala, an alanine side chain (to represent any of the non-glycyl amino acids that commonly occur in proteins) was affixed to each of the linking α-carbon atoms for R_2 and R_3 [Fig. 2 (*a*)]. Since *cis*-prolines are observed in polypeptides like polyproline I [9] and in globular proteins like ribonuclease [10], thermolysin [11], subtilisin [12],

*We follow throughout this paper the latest conformational nomenclature [8] in which all dihedral angles are zero for the *cis* conformation.

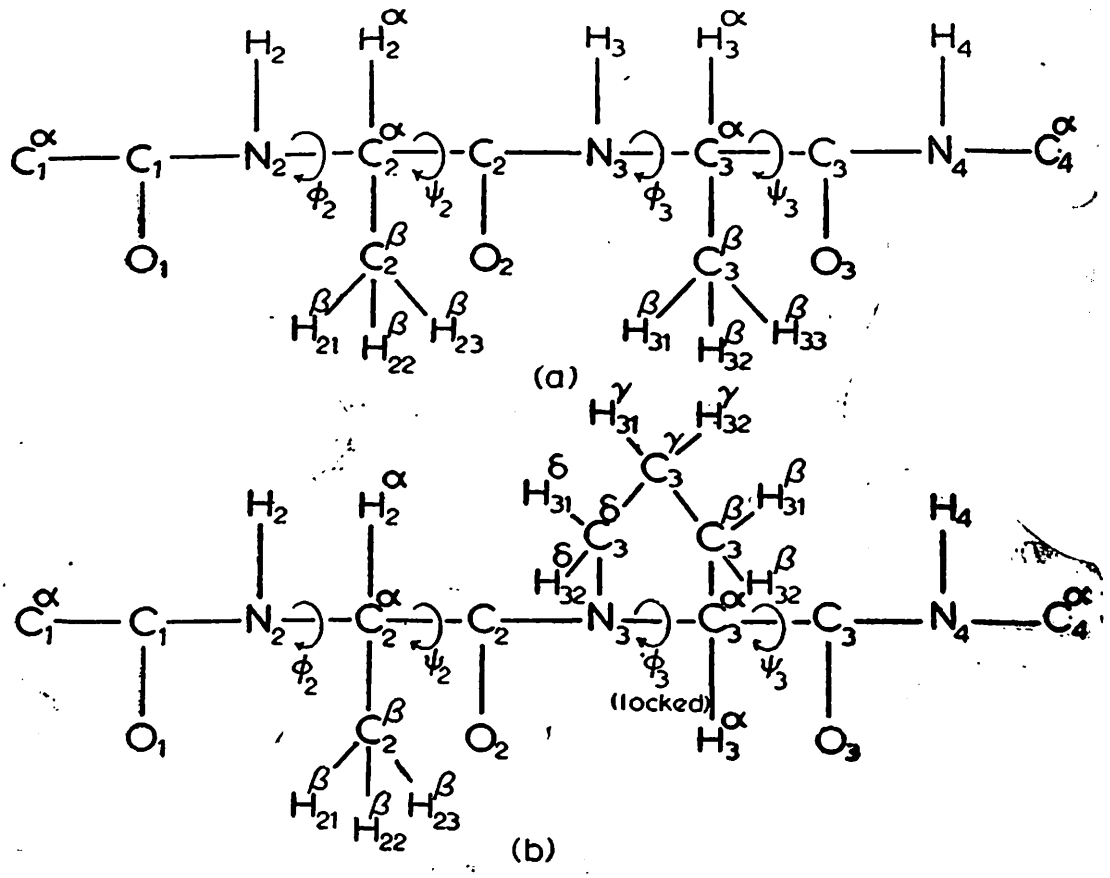

FIG. 2. (*a*) The tripeptide fragment Ala-Ala employed in the calculations for which the four dihedral angles ϕ_2, ψ_2; ϕ_3, ψ_3 are the variables. Atoms from C_1^{α} to C_3^{α} constitute the linked dipeptide where ϕ_2 and ψ_2 are the variable dihedral angles.

(*b*) The Ala-Pro tripeptide fragment used in the calculation. Since ϕ_3 is fixed by the pyrrolidine ring geometry, only ϕ_2, ψ_2 and ψ_3 are variables.

erythrocruorin [13] and carbonic anhydrase [14], in the second set of calculations R_3 was taken to correspond to a prolyl residue instead of being an alanyl residue, so that the tripeptide unit Ala-Pro [Fig. 2 (*b*)] was analysed. These two were expected to reveal from their respective probabilities of occurrence evaluated from the calculated thermodynamic functions, how non-prolyl and prolyl residues would behave in a long-chain polypeptide in *cis* and *trans* conformation of the peptide unit.

CALCULATIONS

Dipeptide L-Ala.—For the dipeptide configurations, the two dihedral angles ϕ and ψ at the linking α-carbon atom were varied in steps of 10° to generate the various conformations.

Tripeptide Ala-Ala.—Different conformations for the tripeptide fragment were generated by varying the dihedral angles ϕ_2, ψ_2, φ_3 and ψ_3 [Fig. 2 (*a*)] in steps of 30° (a larger interval to reduce the otherwise large computer times) throughout the entire range from — 180° to 180°.

Tripeptide Ala-Pro.—Here ϕ_3 is locked because of the closure of the pyrrolidine ring, so that only ϕ_2, ψ_2 and ψ_3 [Fig. 2 (*b*)] are variables. These were varied in steps of 20°. Since the pyrrolidine ring is flexible, it can assume different puckered structures. Three representative puckerings of the pyrrolidine ring were chosen for the T- and C-configurations. The first puckering for the T-configuration was the conformation 1 of the type B characterised by the dihedral angles $\theta = -10°$, $\chi^1 = 25°$, $\chi^2 = -31°$, $\chi^3 = 24°$, $\chi^4 = -8°$, as given by Ramachandran *et al.* [15], while that for the C-configuration was the conformation for *cis*-Pro as observed in the crystal structure of Gly-Pro-Leu [16]. This pucker is characterised by $\theta = -13°$, $\chi^1 = 28°$, $\chi^2 = -33°$, $\chi^3 = 24°$ and $\chi^4 = -6°$ and is close to the first puckering employed for the T-configuration calculation. The conformation of the pyrrolidine ring for the second and third puckered structures were the same for both the T- and C-configurations. These were, the conformations 1 and 3 of the type A given by Ramachandran *et al.* [15] and are characterised by $\theta = 5°$, $\chi^1 = -23°$, $\chi^2 = 32°$, $\chi^3 = -28°$ and $\chi^4 = 14°$ and by $\theta = 10°$, $\chi^1 = -25°$, $\chi^2 = 31°$, $\chi^3 = -24°$ and $\chi^4 = 8°$, respectively.

The total conformational energy was evaluated by adding up the non-bonded, electrostatic, torsional and hydrogen bond energy contributions. The dimensions used or the *trans* peptide unit were the same as those given by Ramachandran and Sasisekharan [17] and those for the *cis* peptide unit were as proposed by Ramachandran and Venkatachalam [18]. We used 6-exp potential functions for the non bonded energy and the partial charges for electrostatic energy as given by Ramachandran and Sasisekharan [17], and by Poland and Scheraga [19], for the atoms of the proline residue. The hydrogen bond energy was calculated by employing the algorithm given by Chidambaram *et al.* [20]. The torsional potential for rotation about N—C^α and C^α—C were calculated by using the 3-fold potential functions $V_\phi = 0{\cdot}3\ (1-\cos 3\phi)$, $V_\psi = 0{\cdot}1(1 + \cos 3\psi)$ used in our laboratory [17].

For the dipeptide fragment, the total potential energy is given by $V = V(\phi, \psi)$, while for the tripeptide fragment, it is $V = V(\phi_2, \psi_2; \phi_3, \psi_3)$. When the middle residue is proline, ϕ_3 is a fixed parameter k and so $V =$

$V(\phi_2, \psi_2; k, \psi_3)$. As mentioned earlier, the *cis* and *trans* forms of the peptide unit differ in their intrinsic energies. Thus, for comparing the all-*trans* sequence with the sequence having a *cis* peptide unit, one must also include the intrinsic energy E in the calculations. In order to correspond to the same basis of V equal to zero, the potential energy V for the configurations having a *cis* peptide unit, namely, *tran-cis* and *cis-trans* in case of dipeptide (Ala) and *trans-cis-trans* in case of tripeptide (Ala-Ala), were obtained by adding the intrinsic energy difference $\triangle E\,(E_{cis}\text{-}E_{trans})$ to the conformational energy composed of the terms mentioned above. The value for $\triangle E$ was taken to be 2·8 kcal/mole. This corresponds to the experimentally observed energy difference between the *cis* and *trans* forms of N-methyl acetamide (*see* Table I), a compound that closely resembles an isolated peptide unit. Since for a prolyl residue, the *cis* and the *trans* forms of the peptide unit are almost identical, the intrinsic energy for the two isomers will be nearly the same. Thus, $\triangle E$ was taken as zero in our calculation for the Ala-Pro tripeptide unit. The partition function Z, the average energy $\langle E\rangle$, and the free energy F were computed by making use of the formulae given below.

For a structure whose conformation is specified by n sets of dihedral angles ϕ_j, ψ_j ($j = 1$ to n) the partition function Z is given by Flory [21] as

$$Z = \int_{\phi_1}\int_{\psi_1}\cdots\int_{\phi_n}\int_{\psi_n} \exp\{-V(\phi_1, \psi_1; \cdots, \phi_n, \psi_n)/RT\} \times d\phi_1, d\psi_1 \cdots d\psi_n \tag{1}$$

Approximating the integrations by summations, we get as indicated by Premilat and Hermans, Jr. [22],

$$Z = \frac{(2\pi)^n}{P} \sum_{\phi_1}\sum_{\psi_1}\cdots\sum_{\phi_n}\sum_{\psi_n} \exp\{-V(\phi_1, \psi_1, \cdots, \phi_n, \psi_n)/RT\} \tag{2}$$

where P is the number of points in the n-dimensional conformational phase space at which the conformatonal energy V is evaluated. Similarly, the average energy $\langle E\rangle$ is approximated by

$$\langle E\rangle = \frac{(2\pi)^n}{PZ} \sum_{\phi_1}\sum_{\psi_1}\cdots\sum_{\phi_n}\sum_{\psi_n} V(\phi_1, \psi_1, \cdots, \phi_n, \psi_n) \times \exp\{-V(\phi_1, \psi_1, \cdots, \phi_n, \psi_n)/RT)\}. \tag{3}$$

For the dipeptide system, the partition function Z and the average energy $\langle E \rangle$ will then be given by

$$Z = \frac{4\pi^2}{P} \sum_{\phi} \sum_{\psi} \exp\{-V(\phi, \psi)/RT\} \tag{4}$$

$$\langle E \rangle = \frac{4\pi^2}{PZ} \sum_{\phi} \sum_{\psi} V(\phi, \psi) \exp\{-V(\phi, \psi)/RT\} \tag{5}$$

while, for the tripeptide system, these will be given by

$$Z = \frac{16\pi^4}{P} \sum_{\phi_2} \sum_{\psi_2} \sum_{\phi_3} \sum_{\psi_3} \exp\{-V(\phi_2, \psi_2; \phi_3, \psi_3)/RT\} \tag{6}$$

$$\langle E \rangle = \frac{16\pi^4}{PZ} \sum_{\phi_2} \sum_{\psi_2} \sum_{\phi_3} \sum_{\psi_3} V(\phi_2, \psi_2; \phi_3, \psi_3) \exp\{-V(\phi_2, \psi_2; \phi_3, \psi_3)/RT\} \tag{7}$$

The free energy F in either case can be calculated from the relation $F = -RT \ln Z$. If Z_C and Z_T denote the partition functions, for the dipeptide (or tripeptide) configuration containing a *cis* peptide unit and that for the corresponding all-*trans* sequence, respectively, the probability of occurrence p_C of a *cis* peptide unit is given by

$$p_C = Z_C / (Z_T + Z_C). \tag{8}$$

For the Ala-Ala system, calculations were also performed by taking a different set of torsional potential functions proposed recently by Kolaskar *et al.* [23, 24] which has the form $V_\phi = 0$, $V_\psi = 2{\cdot}0\ (1-\cos 2\psi)$ and also by varying the bond angle τ (N—C$^\alpha$—C) from the standard value of 110° to a larger value of 114°.

Results and Discussion

1. *Dipeptide system*

The conformational energy calculation on the dipeptide configurations revealed that the minimum energy of the *trans-trans* system was $-2{\cdot}0$ kcal/mole for $\phi = -90°$, $\psi = 80°$, when no hydrogen bond energy was considered and $-3{\cdot}6$ kcal/mole for $\phi = -90°$, $\psi = 60°$, when the energy contribution from the $2{\cdot}2_7$-type internal hydrogen bond [25], was included. These minimum-energy values were much lower than the minimum energies of the *trans-cis* and *cis-trans* configurations which were $1{\cdot}6$ kcal/mole for

$\phi = -140°$, $\psi = 150°$, and 2·1 kcal/mole for $\phi = -140°$, $\psi = 155°$ respectively. Due to the presence of the *cis* peptide unit, the formation of an internal dipeptide hydrogen bond (as in the case of *trans-trans* dipeptide) is prevented, so that, in the case of *cis-trans* and *trans-cis* configurations, lowering of energy through the formation of a hydrogen bond is not possible. We also observed that the presence of a *cis* peptide unit reduces the extent of the low energy regions of the dipeptide unit. This can be clearly seen from the energy contour diagrams (Fig. 3). As a result of this, the average

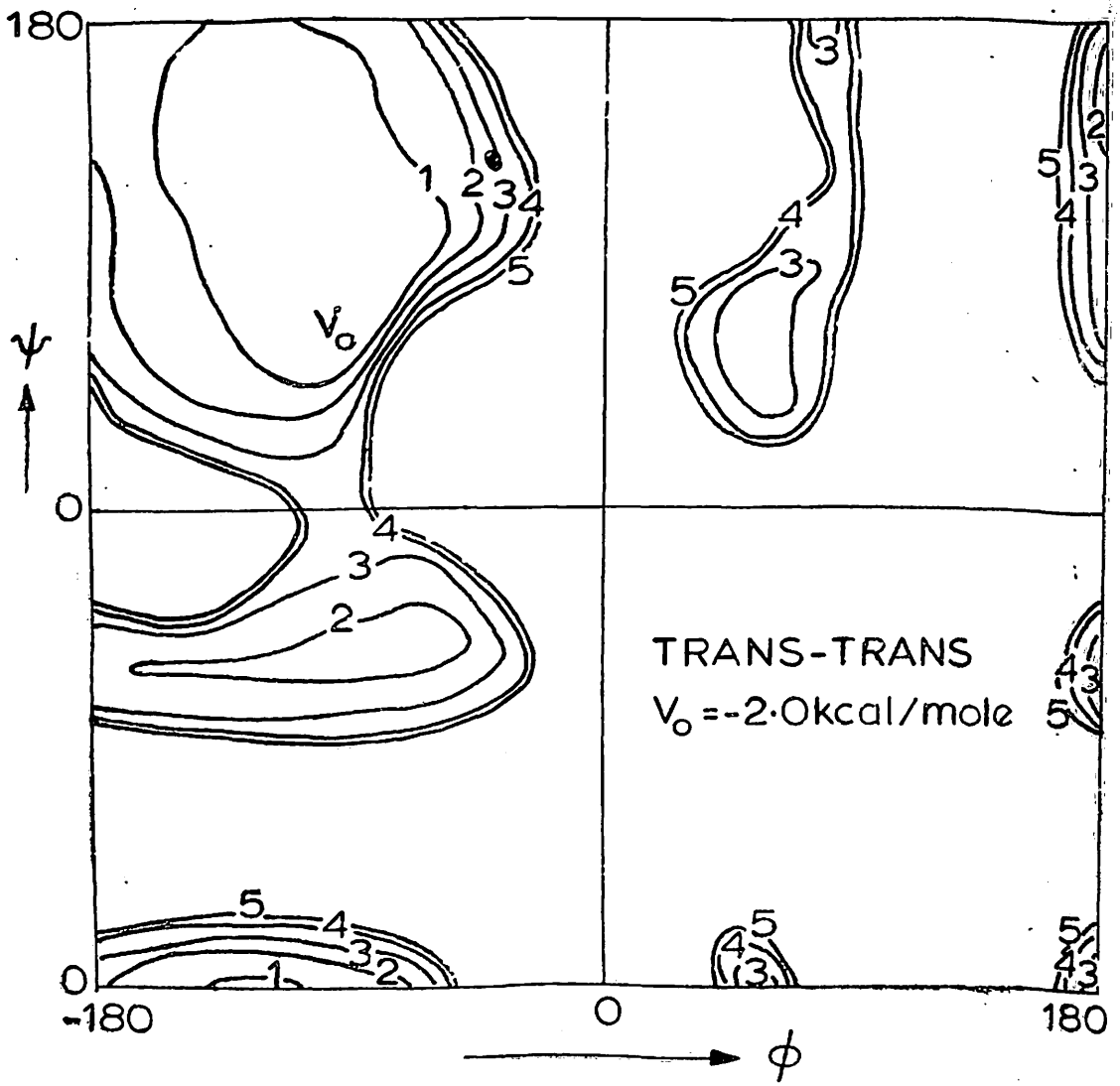

FIG. 3. (*a*) The low energy contours of 1, 2, 3, 4, and 5 kcal/mole above the minimum energy for the *trans-trans* dipeptide configuration. No hydrogen bond energy is included The minimum energy conformation is marked by *X*, and the absolute value of the minimum energy (V_φ) is given in the lower right hand quadrant.

energy and the free energy of the *trans-trans* configuration are lower by about 4 kcal/mole, while the partition function is about 300 times higher than that for either *trans-cis* or *cis-trans* configuration. The probability p_c of *cis* peptide occurrence turns out to be about 0·003 for this case (*see* Table II). We thus find that, even in a dipeptide, a *cis* peptide unit reduces the thermodynamical stability and the probability of the structure.

2. *Tripeptide system*

A more realistic representation, *vis-a-vis* the situation in a polypeptide chain, exists in a tripeptide, which can be expected to mimic a polypeptide, since it accounts for all the short-range interactions that dominate and influence the conformation of a long polypeptide chain.

Ala-Ala case.—For the Ala-Ala system, we obtained results which were qualitatively similar to those in the case of the dipeptide, *i.e.*, here also the calculations indicated that the presence of a *cis* peptide unit introduces appreciable conformational inflexibility. Thus, it was observed that the region of allowed conformation for the T-configuration is very much more than that of the C-configuration and that most of the disallowed conformation in the latter case had short contacts between the two terminal *trans* peptide units. This feature is illustrated in Fig. 4 which shows sample conformations for the two tripeptide configurations. Although the conformation of *trans-cis* and the *cis-trans* dipeptide fragment occurs in the steri-

TABLE II

Thermodynamic parameters of the dipeptide configurations, and the probability of occurrence of cis *peptide unit*

Thermodynamic quantities	Partition function Z	Average energy $\langle E \rangle$ kcal/mole	Free energy F kcal/mole	Probability of occurrence of *cis* peptide units p_c*
Dipeptide configurations				
trans-trans	1313·0	—1·4	—2·2	..
trans-cis	4·4	2.2	1·4	$3{\cdot}3 \times 10^{-3}$
cis-trans	3·5	2·5	1·6	$2{\cdot}7 \times 10^{-3}$

* See text, in the section "Calculations" for the definition.

cally allowed region, Fig. 4 (*b*) shows that the tripeptide moiety *trans-cis-trans* possesses a completely disallowed conformation as the terminal *trans* peptide units are in very close proximity across the middle *cis* peptide unit.

The minimum energy for the T-configuration was found to occur at $\phi_2 = -90°$, $\psi_2 = 60°$; $\phi_3 = -90°$, $\psi_3 = 60°$, having the energy values of $-7{\cdot}9$ kcal/mole and $-7{\cdot}2$ kcal/mole respectively for the two cases, namely, for τ equal to 110° (set one) and for τ equal to 114° (set two), with

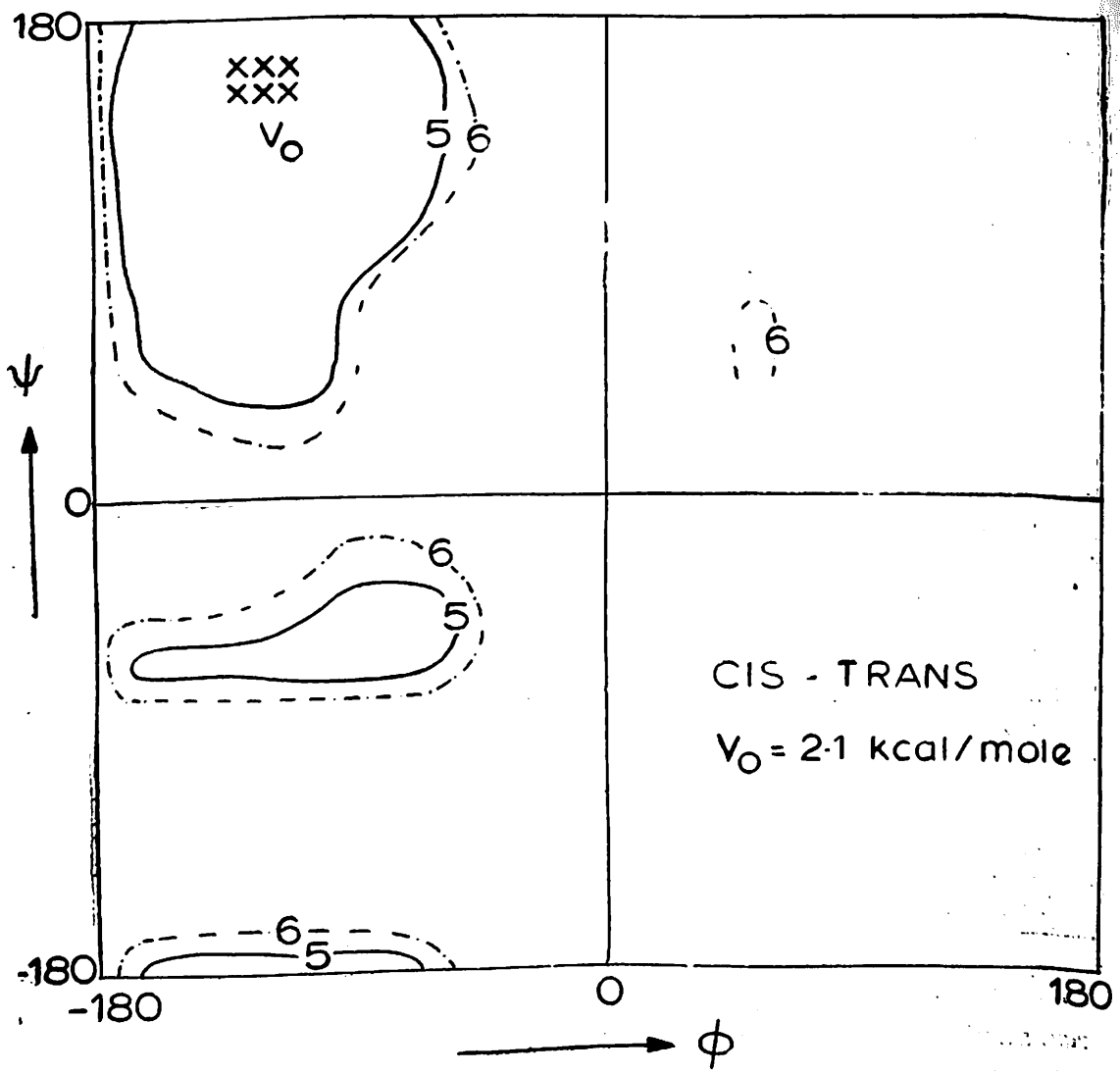

FIG. 3. (*b*) The low energy contours for the *cis-trans* pipeptide configuration, drawn relative to the minimum energy of the *trans-trans* configuration. The minimum energy conformations at 10° intervals, for this configuration, are marked by *X* and their value (V_0) is also given in the lower right hand quadrant.

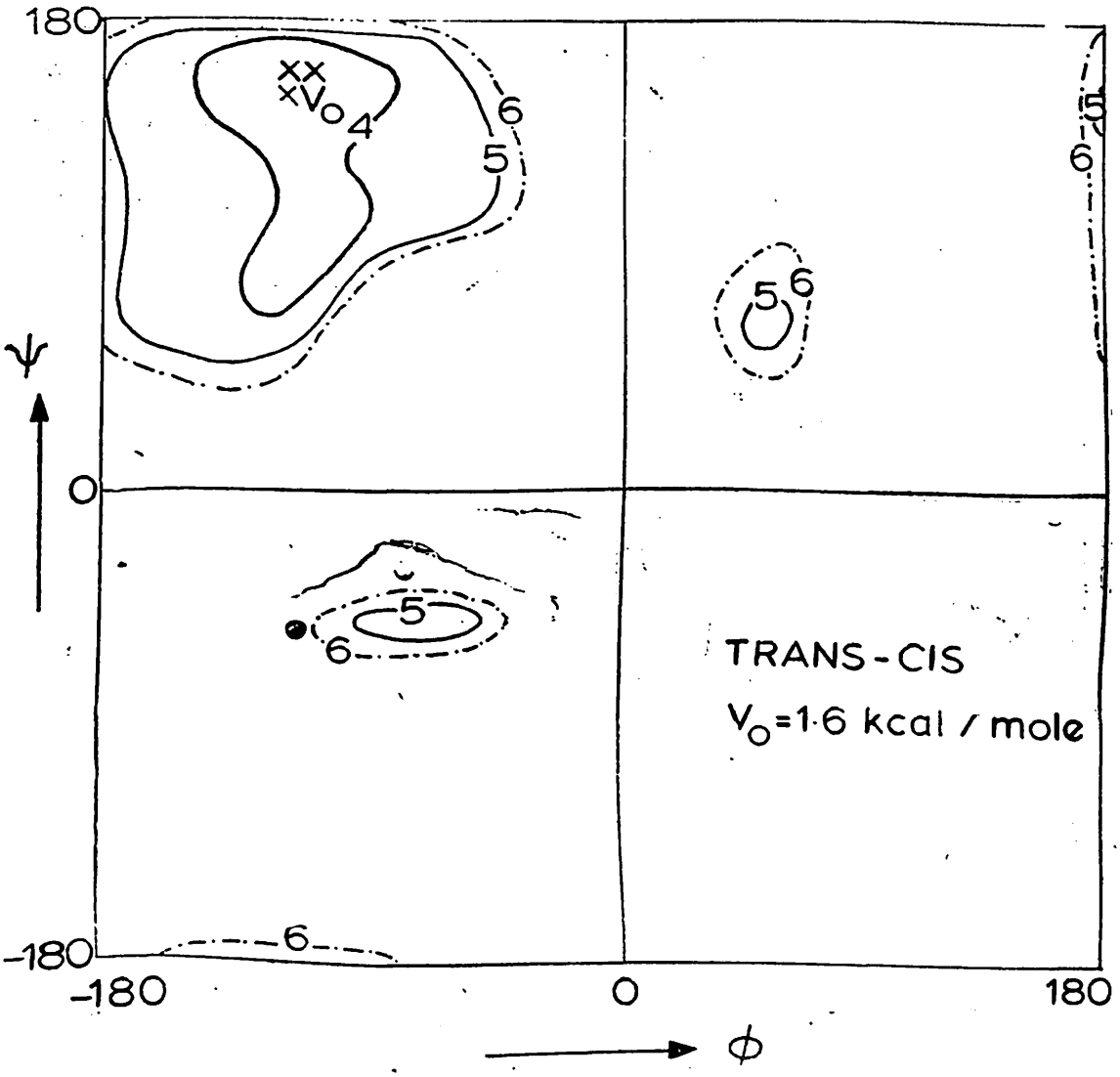

FIG. 3. (*c*) The low energy contours for the *trans-cis* depeptide configuration, drawn relative to the minimum energy of the *trans-trans* configuration. The minimum energy conformations for this configuration are marked by *X*, and their value (V_o) is also given in the lower right hand quadrant.

the 3-fold torsional potential functions for ϕ and ψ. These conformations were stabilised through the presence of an internal $2{\cdot}2_7$-type hydrogen bond in the two *trans-trans* dipeptide fragments constituting the T-configuration of the tripeptide system. The C-configuration, on the other hand, had a significantly higher minimum energy. These occurred at $\phi_2 = -120°$, $\psi_2 = 90°$; $\phi_3 = -120°$, $\psi_3 = 120°$, with an energy value of $-3{\cdot}1$ kcal/mole for set one, and at $\phi_2 = -120°$, $\psi_2 = 60°$; $\phi_3 = -90°$, $\psi_3 = 150°$,

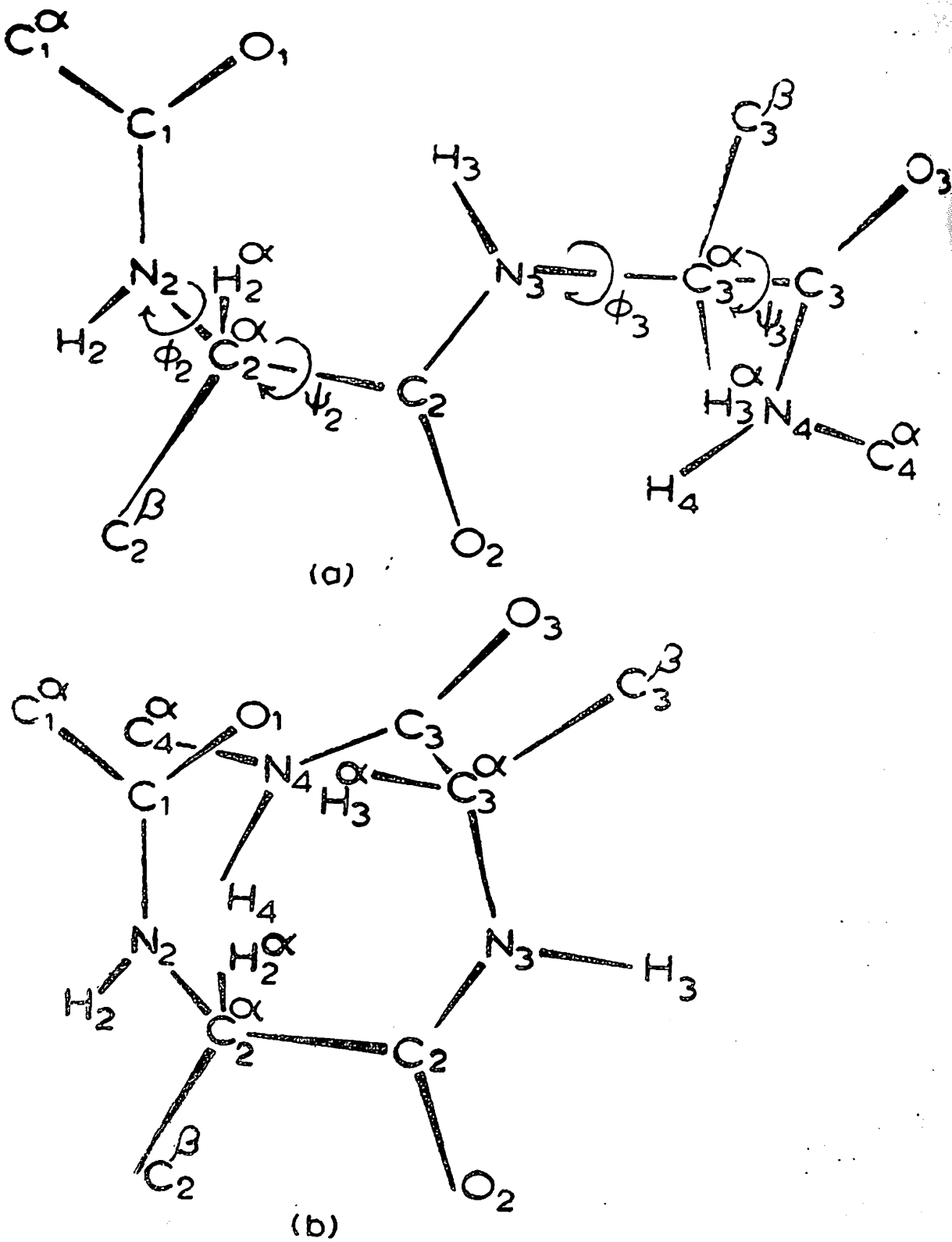

FIG. 4. Schematic drawing of the conformations of (*a*) *trans-trans-trans* and (*b*) *trans-cis-trans* structure for $\phi_2 = -90°$, $\psi_2 = 60°$; $\varphi_3 = -90°$, $\psi_3 = 60°$. Note that the latter is disallowed by severe short contacts, although the sequence *trans-cis* and *cis-trans* are both allowed.

TABLE III

(a) The low energy conformations for L-Ala-L-Ala ($\tau = 110°$)

T-configuration				
ϕ_2 (°)	ψ_2 (°)	ϕ_3 (°)	ψ_3 (°)	Δv*
— 90	60	— 90	60	0·0
— 90	60	— 90	90	1·8
— 60	150	— 90	60	2·3
—120	150	— 90	60	2·3
— 90	60	—120	60	2·3
— 90	60	—120	90	2·3
— 90	120	— 90	60	2·3
—120	90	— 90	60	2·3
—120	120	— 90	60	2·4
— 90	60	—120	120	2·4
— 90	60	—120	150	2·4
— 60	90	— 90	60	2·4
— 90	60	— 90	120	2·5
—120	60	— 90	60	2·5
		C-configuration		
—120	90	—120	120	4·8
—150	60	— 90	150	5·2
—150	90	— 60	150	5·2
—150	90	— 90	120	5·3
—120	90	— 90	150	5·5
—120	90	— 90	180	5.7
—150	60	— 90	180	5·8
60	60	— 90	— 60	6·2
60	150	— 90	30	6·3

* Δv in kcal/mole is over the minimum energy of — 7·9 kcal/mole for the *T*-configuration, occurring at $\phi_2 = -90°$, $\psi_2 = 60°$; $\phi_3 = -90°$, $\psi_3 = 60°$.

TABLE III

(*b*) *The low energy conformations for L-Ala-L-Ala* ($\tau = 114°$)

T-configuration				
ϕ_2(°)	ψ_2(°)	ϕ_3(°)	ψ_3(°)	Δ_V*
— 90	60	— 90	60	0·0
— 30	—60	— 90	60	1·2
— 90	90	— 90	60	1·3
—120	150	— 90	60	1·5
— 60	90	— 90	60	1·5
—150	150	— 90	60	1·6
— 30	—60	—120	30	1·6
— 60	—60	— 90	60	1·7
—120	90	— 90	60	1·7
—120	90	— 90	60	1·8
—120	120	— 90	60	1·8
— 90	60	— 90	90	1·8
— 90	150	— 90	60	1·8
— 60	120	— 90	60	1·9
—150	180	— 90	60	2·0
—150	120	— 90	60	2·0
—120	180	— 90	60	2·0
—120	60	— 90	60	2·0
— 30	—60	—120	60	2·1
— 60	150	— 90	60	2·1
— 60	150	— 90	60	2·1
— 90	30	— 90	60	2·2

TABLE III (b) (*Contd.*)

—150	90	— 90	60	2·3
— 90	180	— 90	60	2·3
— 90	—60	— 90	60	2·3
60	180	— 90	60	2·5
— 90	60	— 90	150	2·5
— 90	60	— 90	120	2·5
— 90	60	—120	150	2·5
		C-configuration		
—120	60	— 90	150	5·3
— 60	150	— 90	30	5·9
—120	60	— 90	180	6·3
— 90	120	—120	90	6·3

*Δv in kcal/mole, is over the minimum energy of — 7·2 kcal/mole for the T-configuration, occurring at $\phi_2 = -90°$, $\psi_2 = 60°$; $\phi_3 = -90°$, $\psi_3 = 60°$.

with an energy value of — 1·9 kcal/mole for set two. These conformations had a chain-reversing type of structure for the tripeptide fragment, and a weak hydrogen bond was noted between the first and the third *trans* peptide units. It was observed that the low energy region for the C-configuration was much more restricted than that of T-configuration, as can be seen from Table III, where the low energy conformations for both the T- and C-configurations are listed at 30° intervals, at which calculations were performed. This interval is coarse, but was chosen to avoid very large computer time. Because of the large grid size, the low energy β bend conformations for the L-Ala-L-Ala tripeptide [26] having 4→1 hydrogen bond were not generated and so are not presented in Table III (*a*).

Since the low energy region for the C-configuration is small, it has much higher average energy in each of the two sets of calculations as can be seen

from Table IV. This clearly indicates that the presence of a *cis* peptide unit in the C-configuration causes the same to assume on an average a conformation of higher energy than that for the all-*trans* T-configuration. Calculation of the partition function showed that this was higher, *i.e.*, the thermodynamic probability was larger, for the T-configuration compared to that of the C-configuration by a factor of 1100 and 5400 respectively, for the sets one and two respectively. As mentioned earlier, the 30° grid size employed in the calculation did not include the β bend regions for the tripeptide unit Ala-Ala. Also the rapidly varying low energy conformations around the $2{\cdot}2_7$ hydrogen bonded regions were not fully generated. On including the contributions from these two regions through a closer search (at intervals of 10° for the dihedral angles around the minimum energy conformation) employing 10° grid size, the calculated value of probability of *cis* peptide occurrence reduces to 0·0005 (*see* Table IV) from 0·0009 for the 30° search. The T-configuration was found to be thermodynamically more stable as its free energy turned out to be lower by about 4·3 kcal/mole in the two sets (*see* Table IV). A similar comparative trend in the values of the thermodynamic quantities was observed even when calculations were performed with an entirely different torsional potential (2-fold ψ-potential) mentioned earlier, with τ retained at 110° (set three). For this set, the minimum energy of the T- and C-configuration occurred for $\phi_2 = -60°$, $\psi_2 = -30°$, $\phi_3 = -120°$, $\psi_3 = 30°$ with an energy value of $-3{\cdot}7$ kcal/mole and for $\phi_2 = -60°$, $\psi_2 = 150°$; $\phi_3 = -90°$, $\psi_3 = 30°$ with an energy value of $-0{\cdot}7$ kcal/mole respectively. These minimum energy conformations were distinct from those observed for the first two sets and also the energy values were quite close. But here also, calculations revealed that the T-configuration had a higher partition function, a lower average energy and a lower free energy, although for each thermodynamic quantity the difference in their values for the two configurations are not so marked as in the first two sets (*see* Table IV). For this set, the partition function for either of the two configurations is lower, while the average energy and the free energy are higher, mainly because of the higher positive contribution to the conformational energy for the 2-fold ψ-potential, which has a 4·0 kcal/mole barrier, compared to only 0·2 kcal/mole for the 3-fold ψ-potential.

Summing up, our results indicate that in so far as a tripeptide fragment is a good model for a polypeptide, a *cis* peptide unit in the middle of an otherwise all-*trans* polypeptide chain introduces severe stereochemical restrictions so that the low population of *cis* peptide unit can, to a good extent, be explained on the basis of this consideration. In fact, as

TABLE IV

The partition function Z, the average energy ⟨E⟩ and the free energy F for the Ala-Ala system. T stands for trans-trans-trans *configuration and C stands for* trans-cis-trans *configuration*

Thermodynamic quantity	Z_T	Z_C	$\langle E\rangle_T$ kcal/mole	$\langle E\rangle_C$ kcal/mole	F_T kcal/mole	F_C kcal/mole	p_c[a]
Set of calculation							
1							
($\tau = 110°$, 3-fold potential)	[b] 75540.0	69.0	−6y67	−2·12	−6·75	−2·5	$9·0\times10^{-4}$
	[c] 137075.0	69·0	−6·85	−2·12	−7·05	−2·5	$4·5\times10^{-4}$
2							
($\tau = 114°$, 3-fold potential)	40330·0	7·6	−5·55	−0·41	−6·37	−1·18	$1·85\times10^{-4}$
3							
($\tau = 110°$, 2-fold potential)	401·0	0·86	−3·0	−0·39	−3·51	−0·13	$20·0\times10^{-4}$

[a] p_c is the probability of the occurrence of *cis* peptide unit; see text in the section "Calculations", for the definition.

[b] Calculations with 30° grid size for the dihedral angles.

[c] Calculation for T-configuration with 30° grid size, except at the β-bend regions [26] and at the $2·2_7$ hydrogen bonded regions where 10° grid size was employed—see text in the section calculations.

can be seen from the last column of Table IV, the probability p_c of *cis* peptide occurrence obtained on the basis of the tripeptide calculation is very small. Thus we have shown that the extremely low value of *cis* peptide population as observed in protein structures can be arrived at theoretically by including the effects of stereochemical inflexibility produced by the introduction of a *cis* peptide unit in an all-*trans* polypeptide chain together with the intrinsic energy difference between the *cis* and *trans* peptide units.

Ala-Pro case.—The situation, however, is quite different when the second amino acid residue corresponds to that of proline. Calculations on the tripeptide fragment Ala-Pro yield results of different trend to that for the Ala-Ala case. Thus, unlike in the Ala-Ala the case, the C-configuration has lower minimum energy than the T-configuration. The minimum energy of the C-configuration occurs at $\phi_2 = -140°$, $\psi_2 = 80°$ and $\psi_3 = 160°$ with $\phi_3 = -71{\cdot}7°$ (This value for ϕ_3 corresponds to that for a *cis*-prolyl residue in Gly-L-Pro-L-Leu), with an energy value of $-10{\cdot}4$ kcal/mole which is 2·0 kcal/mole lower than the minimum energy for the T-cofiguration occurring at the conformation defined by $\phi_2 = -120°$, $\psi_2 = 80°$ and $\psi_3 = 100°$ with $\phi_3 = -70°$ corresponding to *trans* prolyl residue in conformation 1 of type B in Ramachandran *et al.* [15]. Since the NH hydrogen of the second peptide unit does not exist when we have a proline ring as the side chain at the third α-carbon atom, and also because of the closure of the side chain with the backbone locking up ϕ_3 at a value close to $-60°$, the T-configuration of Ala-Pro cannot have the internal $2{\cdot}2_7$ hydrogen bond, as in the Ala-Ala case, resulting in a different minimum energy conformation from that observed for Ala-Ala. For the C-configuration, the minimum energy conformation has an open reverse turn [27] with the two *trans* peptide units on either side facing each other and about parallel to each other with their planes almost normal to the plane of the middle *cis* peptide unit. Such *cis* proline bends have been frequently observed in globular protein structures whenever a *cis* proline is present [28]. The very low energy of this conformation is due to the highly favourable non-bonded and dipole interaction between the nearly parallel terminal *trans* peptide units. This feature, however, does not exist for the T-configuration for which the minimum energy conformation corresponds to the two flanking *trans* peptide units being on the opposite sides of the middle *trans* peptide unit. The average energy of the T-configuration is higher than that of the C-configuration for all the three puckerings of the pyrrolidine ring (*see* Table V). On the other hand, the partition functions Z_C and Z_T are strongly dependent on the pyrrolidine pucker. Thus, while Z_C is larger than Z_T for puckering 1 and 2, it is smaller than Z_T for puckering 3 (*see* Table V). As a result, the probability of *cis* peptide occurrence p_C varies significantly depending on the pyrrolidine pucker. The partition functions for the Ala-Pro case for the two configurations were computed by using the formula

$$Z = \frac{8\pi^3}{P'} \sum_{\phi_2} \sum_{\psi_2} \sum_{\psi_3} \exp\{-V(\phi_2, \psi_2; k, \psi_3)/RT\} \qquad (9)$$

TABLE V

The partition function Z, the average energy ⟨E⟩ and the free energy F for the T- and C-configuration of Ala-Pro tripeptide unit

Set of calculation	Z_T	Z_C	$\langle E\rangle_T$	$\langle E\rangle_C$	F_T	F_C	p_c[a]
1	$8\cdot95 \times 10^5$	$9\cdot78 \times 10^6$	−6·90	−9·30	−8·17	−9·60	0·90
2	$2\cdot08 \times 10^5$	$7\cdot96 \times 10^5$	−6·35	−9·01	−7·30	−8.10	0·79
3	$1\cdot24 \times 10^5$	$0\cdot50 \times 10^5$	−6·04	−7·41	−6·99	−6.45	0·29

* p_c is the probability of *cis* peptide occurrence—see text in the section "Calculations." 1, 2, 3 correspond to different puckerings of the pyrrolidine ring adopted from Yamane *et al.* [18] and Ramachandran *et al.* [17]—see text, in the section "Calculations".

where, because of ϕ_3 being fixed (say at a value k) due to the presence of a prolyl ring at the third α-carbon atom, summation is made only over the remaining three variable dihedral angles ϕ_2, ψ_2 and ψ_3. Here P' is the appropriate number of points in the conformational space at which energy calculation is made in this case.

The results in Table V indicate that a prolyl *cis* peptide unit is thermodynamically at least as favourable as the *trans* form. This is in stark contrast to the case of alanyl residues where, as we have seen, the *cis* conformation of the peptide unit has a very low probability of occurrence. For an elucidation of this distinctive feature of the Ala-Pro system, we studied the Ala-Ala tripeptide unit with ϕ_3 fixed at $-60°$ (close to the value of ϕ for prolyl side chain). It was found that, when ϕ_3 is locked at this value, the partition function of the C-configuration is of the same order as that of the T configuration. Thus, a *cis* peptide unit in case of prolyl side chain possesses a much higher probability of occurrence than for the case of alanyl side chain mainly because the interactions with the neighbouring *trans* peptide units are almost as favourable for a *cis* peptide unit as these are for the *trans* peptide unit.

CONCLUSION

Dipeptides.—Our analysis of dipeptide configurations shows that a *trans* peptide unit is markedly favoured over a *cis* peptide unit. This result is in good agreement with experimental observations which indicate that,

in open chain dipeptides, non-prolyl *cis* peptide units are a rarity and these are present only in cyclic diketopiperazines because of the ring closure restrictions. Among the very few experimental studies conducted in connection with the exploration of *cis-trans* isomerism in non-prolyl dipeptides is a careful infrared study [29] on the compounds, glycyl leucine, leucyl glycine, glycyl phenylalanine and phenylalanineglycine in the amide I (*trans* band at 1655 cm^{-1}, *cis* band at 1690 cm^{-1}) and the amide II and amide III bands. This study indicated that a mixture of *cis* and *trans* peptide linkage is present in aqueous solution and that the *cis* isomer population increases with dilution. Proton NMR studies on protected dipeptides N-benzyloxycarboxyl-L-Ala-L-Ala-O-Me and Boc-L-Ala-L-Ala-O-Me [30] have also revealed *cis-trans* isomerism between the protector group Z (N-benyzloxycarboxyl) or Boc and the first alanine residue. For imino acids like proline, however, NMR and infrared studies have clearly indicated that the *cis* conformation is frequently observed. Thus, ^{13}C NMR studies on the dipeptides Gly-Pro, Ala-Pro and Val-Pro by Thomas and Williams [31] have shown the existence of 2:3 *cis-trans* mixture for the X-pro peptide linkage in aqueous solution. Other studies, employing proton NMR methods, on proline derivatives like N-acetyl-L-proline [32] and N-acetyl-L-proline-N-methyl amide [32, 33] have indicated the presence of about 25%, 25% and 33% respectively, of *cis* peptide units for the peptide bond preceding proline. ^{13}C NMR studies carried out by Deslauriers *et al.* [34] also on such proline derivatives, have yielded a similar *cis-trans* ratio.

Tripeptides and larger peptides.—There is, apparently, no experimental evidence for the existence of *cis* peptide units for non-prolyl residues in tripeptides or larger peptides. Our result, which predicts a very small population for the *cis* form can thus be seen to be in good agreement with experimental observations. However, it must be noted that the predicted probability is not negligible. It is thus likely that *cis* peptide units are not improbable and would occur in some rare cases, On the other hand, for proline-containing peptides, *cis-trans* isomerism has been frequently observed for the X-Pro peptide unit both in solution and in the solid state. In fact, the crystal structure study of Gly-L-Pro-L-Leu [16] has shown for the first time the existence of *cis*-proline in the crystalline state. Among the examples of *cis-trans* isomerism in solution for proline-containing open-chain peptides are the ones observed for the derivatives of the side chain of oxytocin [35] such as S-benzyl-L-Cys-L-Pro-L-Leu-Gly(NH_2) which shows 33% of the *cis* isomer for the Cys-Pro bond and Z-L-Pro-L-Leu-Gly(NH_2) which shows about 50% of *cis* form for the *Z* (N-benzyloxycarboxyl)-Pro bond.

Other studies on small proline-containing peptides like Angiotensin II [36] and T.R.F. [37] show that the *cis* isomer for the His-Pro bond is present to the extent of 20% and 15% respectively, in aqueous solutions, while studies on polypeptides like poly (L-Pro-Gly) and poly (Gly-Gly-L-Pro-Gly) [38] show about 15% *cis* conformation for the Gly-Pro bond. These experimental observations indicate that, although the *trans* conformation in the sequence X-Pro is still a major component in the *cis-trans* isomerism for the peptide unit, the *cis*-conformation is not a rarity but one of frequent occurrence. Our results for the tripeptide unit Ala-Pro, to a large extent, corroborates this observation, although for puckers 1 and 2 employed in the calculation, the results indicate that *cis* form is more probable. In fact, there exist experimental evidences, such as for N-acetyl L-proline, N, N-diisopropyl amide [32] and H-L-Thre-L-Phe-L-Pro-OH [39], in which *cis*-Pro has been observed to have a higher population (about 60%). A noteworthy feature of our calculation on Ala-Pro is that a *cis*-prolyl residue, very much unlike in the non-prolyl case, can provide considerable conformational stability locally in a polypeptide chain. This is due to the low value of the free energy of the tripeptide segment *trans-cis-trans* (configuration C). Thus, a frequent presence of *cis*-prolyl residue in polypeptides and proteins can be confidently expected. It may be mentioned here that we have not investigated the special case when X is also proline, in the fragment X-Pro. Incidentally NMR observations [32] on Pro-Pro indicate that about 35% *cis* isomer is present. However, ^{13}C NMR studies show that increase in chain length of the proline polypeptide to 4 or more causes the percentage of *cis* linkage to decrease drastically [40].

The results reported in this paper show that the population of *cis* peptide units in a polypeptide chain is expected to be small, of the order of 1 in 2000; but there is no reason to believe that they are forbidden, except when associated with Pro. In view of this, we suggest that, *cis* peptide units should be looked for in protein structures, as they are expected to occur in some rare cases. In addition, for the sequence X-Pro, the population of *cis* peptide units is likely to be much more than what has been observed hitherto in globular proteins. Therefore, a closer examination of electron density maps around proline residues in protein crystal structures might be advisable for locating *cis* peptide linkages in such cases (*see* also Huber and Steigemann [28]).

ACKNOWLEDGEMENT

The author is grateful to Prof. G. N. Ramachandran for suggesting the problem and for his constant guidance and help. He thanks Dr. R.

Chandrasekaran for his many constructive criticisms. Thanks are due to SERC (DST), India, for financial assistance. The author is a recipient of a scholarship from NCERT, India.

REFERENCES

[1] Murthy, A. S. N., Gurudath Rao, K. and Rao, C. N. R. — A molecular orbital study of the configuration, protonation, and hydrogen bonding of secondary amides. *J. Amer. Chem. Soc.*, 1970, **92**, 3544–48.

[2] Pericaudet, M. and Pullman, A. — An *Ab-initio* quantum-mechanical investigation on the rotational isomerism in amides and esters. *Int. J. Peptide Protein Res.*, 1973, **5**, 99–107.

[3] Andrews, P. R. .. — *Cis-Trans* isomerism of the peptide bond. *Biopolymers*, 1971 **10**, 2253–2267.

[4] Shipman, L. L. and Christofferson, R. E. — *Ab-initio* calculations on large molecules using molecular fragments, model peptide studies. *J. Amer. Chem. Soc.*, 1973, **95**, 1408–1416.

[5] Neumann, R. C. Jr., Jonas, V., Anderson, K. and Barry, R. — Hindered rotation in N-methylformamide. A peptide bond model system. *Biochem. Biophys. Res. Comm.*, 1971, **44**, 1156–1161.

[6] Drakenberg, T., Dahlqvist, K. i. and Forsen, S. — The barrier to internal rotation in amides. IV. N, N-dimethylamides; substituent and solvent effects. *J. Phys. Chem.*, 1972, **76**, 2178–2183.

[7] Khetrapal, C. L. .. — Personal communication to Prof. G. N. Ramachandran.

[8] IUPAC–IUB Commission on Biochemical Nomenclature. — *Biochemistry*, 1970, **9**, 3471–3478.

[9] Traub, W. and Shmueli, V. — Structure of poly-L-proline I. *Nature (London)*, 1963, **198**, 1165–1166.

[10] Wyckoff, H. W., Tsernoglou D., Hanson, A. W., Knox, J. R., Lee, B. and Richards, F. M. — The three-dimensional structure of Ribonuclease-S. *J. Biol. Chem.*, 1970, **245**, 305–328.

[11] Matthews, B. W., Weaver, L. H. and Kester, W. R. — The conformation of thermolysin. *J. Biol. Chem.*, 1974 **249**, 8030–8044.

12] Alden, R. A., Birkoft, J. J., Kraut, J., Robertus, J. D. and Wright, C. S. — Atomic coordinates for Subtilisin BPN' (or Novo)., *Biochem. Biophys. Res. Comm.*, 1971, **45**, 337–344.

[13] Huber, R., Epp, O., Steigemann, W. and Formanek — The atomic structure of erythrocruorin in the light of the chemical sequence and its comparisons with myoglobin. *Eur. J. Biochem.*, 1971, **19**, 42–50.

[14] Kannan, K. K., Nostrand, B., Fridborg, K., Lövgren, S., Ohlsson, A. and Petef, M. — Crystal structure of human erythrocyte carbonic anhydrase B. Three-dimensional structure at a nominal 2·2-Å resolution. *Proc. Nat. Acad. Sci. U.S.A.*, 1975, **72**, 51–55.

[15] Ramachandran, G. N., Lakshminarayanan, A. V., Balasubramanian, R. and Tegoni, G. — Studies on the conformation of amino acids, XII. Energy calculations on prolyl residue. *Biochem. Biophys. Acta*, 1970, **221**, 165–181.

[16] Yamane, T., Ashida, T., Kakudo, M. and Sasada, Y. — *Cis*-proline in the linear Oligopeptide: Benzyloxycarbonyl-Gly-Pro-Leu. *Acta Cryst.*, 1975, A31, Part S3, S47.

[17] Ramachandran, G. N. and Sasisekharan, V. — Conformations of polypeptides and proteins. In *Advances In Protein Chemistry*, 1968, **23**, 283–438.

[18] Ramachandran, G. N. and Venkatachalam, C. M. — Stereochemical criteria for polypeptides and proteins. IV. Standard dimensions for the *cis*-peptide unit and confor mation of *cis*-polypeptides. *Biopolymers*, 1968, **6**, 1255–1262.

[19] Poland, D. and Scheraga, H. A. — Energy parameters in polypeptides. I. Charge distributions and the hydrogen bond. *Biochemistry*, 1967, **6**, 3791–3800.

[20] Chidambaram, R., Balasubramanian, R. and Ramachandran, G. N. — Potential functions for hydrogen bond interactions. I. A modified Lippincott-Schroeder potential function for N—H···O interaction between peptide groups. *Biochem. Biophys. Acta*, 1970, **221**, 182–190.

[21] Flory, P. J. .. Chapter 7, In : *Statistical Mechanics of Chain Molecules*, Academic Press, New York, 1969.

[22] Premilat, S. and Hermans, J. Jr. — Conformational statistics of short chains of poly (L-Alanine) and Poly (Glycine) generated by Monte-Carlo method and the partition function of chains with constrained ends. *J. Chem. Phys.*, 1973, **59**, 2602–2612.

[23] Kolaskar, A. S., Sarathy, K. P. and Sasisekharan, V. — The need for a modified ψ-potential in the dipeptide model. *Curr. Sci.* 1975, **44**, 35–38.

[24] Kolaskar, A. S., Sasisekharan, V. and Sarathy, K. P. — A note on the torsional potential function $V(\phi)$ in the dipeptide model. *Theor. Chim. Acta*, 1975, **38**, 109–114.

[25] Donohue, J. .. Hydrogen bonded helical configuration of the polypeptide chain. *Proc. Nat. Acad. Sci. USA*, 1953, **39**, 470–478.

[26] Chandrasekaran, R., Lakshminarayanan, A. V., Pandya, U. V. and Ramachandran, G. N. — Conformation of the LL and LD hairpin bends with internal hydrogen bonds in proteins and peptides. *Biochim. Biophys. Acta*, 1973, **303**, 14–27.

[27] Crawford, J. L., Lipscomb, N. N. and Schellman, C. G. — The reverse turn as a polypeptide conformation in globular proteins. *Proc. Nat. Acad. Sci. USA*, 1973, **70**, 538–542.

[28] Huber, R. and Steigemann, W. — Two *cis*-prolines in the Berce-Jones Protien Rei and the *cis* Pro-bond. *FEBS Letters*, 1974, **48**, 235–237.

[29] Siemion, F. Z. and Sucharda-Sobczyk, A. — On the conformation of dipeptides in aqueous solutions *Tetrahedron*, 1970, **26**, 191–197.

[30] Dimicoli, J. L. and Ptak, M. — Etude Par RMN de Dipeptides de l' Alanine en solution Organique. *Tetrahedron Letters*, 1970, **23**, 2013–2016.

[31] Thomas, W. A. and Williams, M. K. — ^{13}C nuclear magnetic resonance spectroscopy and *cis-trans* isomerism in dipeptides containing proline. *J. Chem. Soc., Chem. Comm.*, 1972, p. 994.

[32] Madison, V. and Schellman, C. G. — Location of proline derivatives in conformational space. I. Conformational calculations; Optical activity and NMR experiments. *Biopolymers*, 1970, 9, 511–567.

[33] Pogilliani, L., Ellenberger M., Valat J. and Bellocq, A. M. — NMR investigations of proline and its derivatives. *Int. J. Peptide Protein Res.*, 1975, 7, 345–360.

[34] Deslauriers, R., Garrigou-Lagrange C. Bellocq, A. M. and Smith, I. C. P. — Carbon-13 nuclear magnetic resonance studies on the thyrotropin-releasing factor and related peptides. *FEBS Letters*, 1973, 31, 59–66.

[35] Hruby, V. J., Brewster, A. I. and Glasel, J. A. — NMR studies on the conformation of derivatives of the side chains of oxytocin: Examples of *cis-trans* isomerism. *Proc. Nat. Acad. Sci. USA*, 1971, 68, 450–453.

[36] Deslauriers, R., Walter, R. and Smith, I. C. P. — ^{13}C nuclear magnetic resonance studies of the conformation of the X-Pro Bond in the oligopeptide hormones, thyrotropin-releasing hormone, lutenizing hormone-releasing factor, angiotensin and melanocyte-stimulating hormone release-inhibiting factor. *Biochem. Biophys. Res. Comm.*, 1973, 53, 244–249.

[37] Montagut, M., Lemanceau, B. and Bellocq, A. M. — Conformational analysis of thyrotropin releasing factor by proton magnetic resonance spectroscopy. *Biopolymers*, 1974, 13, 2615–2629.

[38] Torchia, D. A. and Lyerla, J. R. Jr. — Molecular mobility of polypeptides containing proline as determined by ^{13}C magnetic resonance. *Biopolymers*, 1974, 13, 97–114.

[39] Wüthrich, K. and Grathwohl, C. — A novel approach for studies of the molecular conformation in flexible polypeptides. *FEBS Letters*, 1974, 43, 337–340.

[40] Wüthrich, K. — Personal communication to Prof. G. N. Ramachandran.